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Antioxidant properties assessment of the cones of conifers through the combined evaluation of multiple antioxidant assays

Tamás Hofmann*, Eszter Visi-Rajczi, Levente Albert

University of Sopron, Faculty of Forestry, Institute of Chemistry, Bajcsy-Zsilinszky street 4, 9400, Sopron, Hungary

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ABSTRACT

Wood logging generates considerable amounts of waste biomass (e.g. cones, bark, leaves, and roots), worthwile to use, as a potential source of antioxidants. Polyphenols represent an important type of non-enzymatic antioxidants in plants. Current data in scientific literature regarding the antioxidant content of coniferous cones and the antioxidant concentrations at different ripening stages is limited and have not been investigated in detail yet. Our investigation aimed to implement an ultrasonic extraction method for the assessment and comparison of the antioxidant polyphenol content of conifer cones: six arbitrarily selected taxa (*Cedrus atlantica*, *Larix decidua*, *Picea abies*, *Pinus nigra*, *Pseudotsuga menziesii*, *Tsuga canadensis)* commonly found in Hungary were investigated in the present study, which provides methodology for future investigations. The comparison of green, mature, and opened cones were carried out for each taxon. Folin-Ciocâlteu total phenol content, ferric reducing ability of plasma (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays were used to assess the antioxidant properties. The 30 min ultrasonic extraction using acetone:water 80:20 v/v solution resulted in the optimum yield on total polyphenols, FRAP, and DPPH. Best values were found for green cones, followed by mature, and opened cones for each species. Overall antioxidant power was determined by a scoring system that combined the three assay results. Taxa with the highest scores were *Picea abies* and *Tsuga Canadensis,* which contained high amounts of antioxidants in both green and mature cones and, surprisingly, also in opened cones (*P. abies*). Results provide a basis for future investigation and comparison involving a large number of taxa.

1. Introduction

The by-products of forestry, logging, and timber production (leaves, branches, cones, root, wood bark, etc.) can be a rich source of plant antioxidants that are worthwhile to extract and use (Robbins, 2003; Pietarinen et al., 2006; Dedrie et al., 2015; Bouras et al., 2016; Tálos-Nebehaj et al., 2017). The potential utilization fields of natural antioxidants are broad and include the production of natural food preservatives (Seeram and Heber, 2007; Coté et al., 2011; Gyawali and Ibrahim, 2014; Kobus-Cisowska et al., 2014), healthcare and healthcare-related products (Packer et al., 1999; Dzialo et al., 2016; Watson et al., 2018), natural growth bioregulators (Popa et al., 2002, 2008; Vyvyan, 2002), and food and drink products (Frydman et al., 2005; Sawalha et al., 2009). Special focus has recently been applied to plant extracts due to their reducing (antioxidant), stabilizing, and coating effects (Fahimirada et al., 2019; Ranoszek-Soliwoda et al., 2019; Rolim et al., 2019) in the production of silver nanoparticles.

Conifers are a large group of resinous, cone-bearing trees and shrubs, comprising the order Coniferales of the Gymnosperms. The seven conifer families are sub-classified into 67 genera, which include over 615 living species (Auders and Spicer, 2012). Conifers bear “seed-cones” and “pollen-cones” out of which the female seed-cones are simply referred to as “cones”. Seed cones were the exclusive subject of the present study.

Forest tree cones have been mostly used for seed extraction for the production of forestry propagation material. The edible seeds extracted from stone pine cones (*Pinus pinea* L.) are one of the most important tree nuts widely planted throughout the Mediterranean region (Kemerli-Kalbaran and Ozdemir, 2019). The residual empty (or as referred to later: opened) cones, generated in several dozen or even as much as several hundred tonnes (Aniszewska and Bereza, 2014), are usually burned in an uncompressed state or can be converted to briquettes (Gendek et al., 2018).

Apart from seed production and pine nuts, only a few coniferous taxa have garnered research attention regarding the use of their cone extractives. The cones *Juniperus* spp. have traditionally been used for flavouring purposes (Lesjak et al., 2011), while the cone extracts and essential oils of *Pinus, Thuya,* and *Cedrus* spp. have been used by

* Corresponding author.
E-mail addresses: hofmann.tamas@uni-sopron.hu (T. Hofmann), visine.rajczi.eszter@uni-sopron.hu (E. Visi-Rajczi), albert.levente@uni-sopron.hu (L. Albert).

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traditional medicine in the Mediterranean region and Japan for their various beneficial health effects (e.g. anti-inflammatory, antioxidant, antiseptic, antifungal, antimicrobial, analgesic effects) and for the treatment of hair loss, rheumatism (Süntar et al., 2012; Djouahri et al., 2014), gastric cancer (Watanabe et al., 1995), common cold, urinary and kidney infections, dermatological disorders, bronchitis, pneumonia, and various other illnesses (Lesjak et al., 2011). The cone extracts of *Pinus Parviflora* Sieb et Zucc were shown to be very powerful against HIV and influenza viruses (Nagata et al., 1990) and also have significant antimutagenic and anticancer effects (Nagasawa et al., 1992). Polyphenolic compounds were shown to have the most powerful antioxidant and ascorbyl radical scavenging activity in these extracts (Nagasawa et al., 1992; Satoh and Sakagami, 1996). The diterpenoid compound sugiol, extracted from the cones of *Metasequoia glyptostroboides* Hu et W.C. Cheng possesses a very strong DPPH$^{\bullet}$ radical scavenging activity (Bajpai et al., 2014). The cone extract of *Juniperus sibirica* Burgsdorf. was characterized by high DPPH and FRAP antioxidant power, which was also explained by the high concentration of phenolic compounds (Lesjak et al., 2011). According to Djouahri et al. (2014), the *Tetraclinis articulata* (Vahl) Mast. cone extracts prepared with ethanol:water 70:30 v/v solution showed the best anti-inflammatory properties, which were attributed to the high polyphenol levels. According to Tumen et al. (2012) the dichloromethane extract of the cones of *Cupressus sempervirens* var. *pyramidalis* (L.) showed high acetylcholinesterase inhibition compared to respective leaf extracts, while Süntar et al. (2012) investigated the anti-inflammatory and wound healing effects of the essential oils of *Pinus* spp. by *in vivo* and *in vitro* experiments. A more recent study reports on the orally active immune adjuvenant prepared from *Pinus sylvestris* (L.) cone extracts, which was found to enhance the proliferative phase of a primary T cell response (Bradley et al., 2014). According to the authors certain polyphenylpropanoid polysaccharide complexes are responsible for the measured effects. Recently Tümen et al. (2018) reported on the chemical composition, wound healing and anti-inflammatory effects of maritime pine (*Pinus pinaster* Ait) cone extracts, which have been used in Turkish folk medicine, supplying scientific evidence for the beneficial health effects. Wang et al. (2019) reported on the antioxidant activity, bioaccessibility and physicochemical properties of the polyphenols of *Pinus koraiensis* (Siebold & Zucc.) cones. In fact, according to the latest scientific results, pine cone and pine cone extracts have come into the focus of research lately, because of various useful properties, e.g. being a source as dietary fibers (Kartal and Ozturk, 2016), or basic materials for the production of coagulants (Hussain et al., 2019) and adsorbents (Kupeta et al., 2018; Mtshatsheni et al., 2019) for water purification.

According to these results, cone antioxidants contribute significantly to health and possess valuable nutritional and other effects. However, apart from the presented data, scientific literature lacks systematic research of the antioxidant composition of cones and the assessment of their role as a source of natural antixoidants. Moreover, in the presented examples, researchers rarely documented sample collection times; more specifically, the phenophase of cone maturity. It can only be assumed that investigations were carried out on unopened, ripe cones still bearing seeds within them. To the best of our knowledge, no research assessing the changes in the antioxidant properties during the ripening process of the cones has been conducted this far.

Our aim was to implement an extraction and evaluation method for the assessment and comparison of the antioxidant content of coniferous cones. For this purpose, six arbitrarily selected coniferous taxa commonly found in Hungary were investigated in the present research, with the aim of providing methodology for future systematic investigations and comparisons of other coniferous taxa.

Extraction was done with aqueous ethanol, methanol, and acetone solutions with different time shedules for the green, mature, and opened cones of the investigated taxa using an ultrasonic extraction method. Ultrasonication was chosen because it is faster and usually requires less solvent compared with other methods (e.g. stirring, Soxhlet extraction).

Antioxidant properties were determined by the Folin-Ciocâlteu total phenol content, ferric reducing ability of plasma (FRAP) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays. The evaluation of the overall antioxidant power was accomplished by a scoring system, which combined the results of the three assays, thus also enabling a comprehensive evaluation of the results between various samples with potentiailly different antioxidant compositions.

2. Materials and methods

2.1. Chemicals and reagents

Double distilled water was prepared for the extractions using conventional distillation equipment. Methanol (HPLC grade) was obtained from VWR International (Budapest, Hungary). Gallic acid, ascorbic acid, DPPH, 2,4,6-tripyridyl-S-triazine (TPTZ), iron(III)-chloride, acetic acid, sodium acetate, hydrochloric acid, and sodium carbonate were obtained from Sigma-Aldrich (Budapest, Hungary). Folin-Ciocâlteu reagent was purchased from Merck (Darmstadt, Germany).

2.2. Sample collection and extraction

Samples were collected from the Botanic Garden of the University of Sopron in Sopron (Hungary) between July-October 2018. The tested taxa were Atlas cedar (*Cedrus atlantica* Endl.), European larch (*Larix decidua* Mill.), Norway spruce (*Picea abies* H. Karst.), black pine (*Pinus nigra* J.F. Arnold), Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco), and eastern hemlock (*Tsuga canadensis* (L.) Carrière). Three types of cones were collected: green, mature, and opened cones. The cone maturation process can take place within one growing season (Norway spruce, European larch, Douglas fir, eastern hemlock), or last two or three seasons (black pine, Atlas cedar) (Auders and Spicer, 2012). Green cones were collected in July when cones are nearly at their full size, yet are still green in color. Cones that mature in two or three years were collected in July of last year. Mature cones were collected in August/September when they turned brown in color and their scales began to open. Opened cones were collected in September/October when cones were fully opened and had released their seeds; they were either found on trees or had already fallen to the ground. One selected mature individual of each taxon was sampled. During each sampling occasion, at least 10 cones were collected from different parts of the crown. Samples were immediately put into sealed plastic bags and stored at −20 °C. Prior to extraction, samples were thawed and ground using a coffee grinder. Ultrasonic extraction was conducted using an Elma Transsonic T570 ultrasonic bath (Elma Schmidbauer GmbH, Singen, Germany) as follows: 0.45 g ground sample was homogenized with 45 ml solvent in a 50 ml centifuge tube. The following solvent compositions were used for all samples: acetone:water 80:20 v/v, methanol:water 80:20 v/v and ethanol:water 80:20 v/v. Extraction times were 10, 20, and 30 min. Extractions of all combinations of taxa, solvents, and extraction times were executed. As temperature can have a significant influence on the extraction, it was controlled during the process. Extraction was done in 10 min cycles and the temperature of the ultrasonic bath was 25 °C at the beginning of each cycle. By the end of a cycle, the temperature increased to 29–30 °C. In the case of the 20 and 30 min extractions, two or three consecutive steps were applied, respectively. The initial bath temperature was set at 25 °C at the beginning of each cycle as described above.

2.3. Determination of antioxidant properties

All assays were run in triplicates with the use of a Hitachi U-1500 type spectrophotometer (Hitachi Ltd., Tokyo, Japan) at the respective wavelengths.

Table 1
Total phenol content (mg GAE/g d.w.) of the samples by applying different extraction sovents (A: acetone:water 80:20 v/v; M: metanol:water 80:20 v/v; E: etanol:water 80:20 v/v) and extraction times (10, 20, 30 min). Different superscript letters for the results of a given sample (taxon, tissue) indicate significant differences at $p < 0.01$ level, except for * $p < 0.001$ and ** $p < 0.002$.

Green cones				Mature cones			Opened cones		
Time solvent	10 min	20 min	30 min	10 min	20 min	30 min	10 min	20 min	30 min
Douglas fir (*Pseudotsuga menziesii*)									
A	39.25 ± 2.68^{c}	50.54 ± 1.98^{d}	48.67 ± 0.90^{d}	13.67 ± 1.03^{d}	17.01 ± 1.35^{d}	17.24 ± 0.89^{d}	7.78 ± 0.33^{d}	10.18 ± 0.73^{e}	11.16 ± 0.66^{e}
M	21.37 ± 1.25^{a}	26.66 ± 0.98^{ab}	38.52 ± 4.86^{c}	11.60 ± 0.16^{abc}	12.13 ± 0.68^{bc}	12.86 ± 0.44^{b}	7.20 ± 0.30^{d}	6.85 ± 0.24^{cd}	7.20 ± 0.21^{d}
E	33.94 ± 0.33^{bc}	37.68 ± 1.51^{c}	37.68 ± 1.51^{c}	9.11 ± 0.36^{a}	10.04 ± 0.73^{ac}	9.25 ± 0.25^{a}	2.46 ± 0.59^{a}	4.07 ± 0.37^{ab} *	5.21 ± 0.55^{bc}
Eastern hemlock (*Tsuga canadensis*)									
A	104.58 ± 3.06^{cd}	125.86 ± 1.17^{e}	118.91 ± 2.97^{de}	77.41 ± 1.87^{ab}	77.45 ± 3.84^{ab}	78.05 ± 2.42^{ab}	3.39 ± 0.31^{b}	3.43 ± 0.44^{b}	3.31 ± 0.08^{b}
M	73.63 ± 7.27^{a}	86.91 ± 5.75^{ab}	87.76 ± 5.16^{abc}	67.39 ± 2.16^{a}	98.32 ± 7.67^{c}	87.95 ± 1.59^{bc}	1.78 ± 0.16 ^{a}	1.89 ± 0.02^{a}	1.96 ± 0.03^{a}
E	101.12 ± 4.32^{bc}	101.47 ± 6.68^{bc} *	90.96 ± 4.29^{bc}	71.92 ± 2.57^{a}	72.04 ± 2.30^{a}	71.36 ± 1.50^{a}	3.40 ± 0.10^{b}	1.98 ± 0.01^{a}	1.89 ± 0.05^{a}
Black pine (*Pinus nigra*)									
A	53.89 ± 1.68^{a}	58.06 ± 1.21^{a}	47.17 ± 9.74^{a}	8.72 ± 0.40^{d}	10.08 ± 0.31^{e}	10.63 ± 0.36^{e}	8.45 ± 0.23^{bc}	7.70 ± 0.37^{ab}	9.20 ± 0.34^{c}
M	46.21 ± 0.53 ^{a}	43.91 ± 0.53^{a}	49.67 ± 0.96^{a}	4.90 ± 0.42 ^{ab}	5.92 ± 0.13^{bc}	6.23 ± 0.19^{c}	6.75 ± 0.16^{a}	7.65 ± 0.20^{ab}	7.72 ± 0.35^{ab}
E	45.42 ± 1.30^{a}	48.65 ± 1.06^{a}	50.73 ± 1.52^{a}	4.67 ± 0.12^{a}	5.68 ± 0.55^{abc}	5.24 ± 0.09^{abc}	7.31 ± 0.57^{ab}	7.84 ± 0.14^{ab}	8.43 ± 0.47^{bc}
Atlas cedar (*Cedrus atlantica*)									
A	32.30 ± 1.11^{c}	39.28 ± 0.81^{e}	44.62 ± 0.16^{f}	9.39 ± 0.26^{b}	11.10 ± 1.34^{bc}	12.29 ± 1.20^{c}	5.37 ± 0.14^{b}	5.90 ± 0.23^{b}	6.05 ± 0.51^{b}
M	24.73 ± 0.90^{b}	31.85 ± 0.46^{c}	35.87 ± 0.46^{d}	9.35 ± 0.46^{b}	10.29 ± 0.13^{bc}	10.32 ± 0.29^{bc}	3.17 ± 0.39^{a}	3.29 ± 0.23^{a}	3.93 ± 0.20^{a}
E	18.14 ± 0.84^{a}	27.49 ± 1.00^{b}	30.94 ± 1.13^{c}	3.36 ± 0.16^{a}	3.79 ± 0.25^{a}	4.80 ± 0.34^{a}	3.14 ± 0.09^{a}	3.53 ± 0.09^{a}	3.79 ± 0.28^{a}
Norway spruce (*Picea abies*)									
A	84.89 ± 3.39^{c}	92.23 ± 1.37^{cd}	105.58 ± 7.92^{e}	55.28 ± 2.31^{ab}	63.06 ± 2.96^{cd}	64.64 ± 2.68^{cd}	27.54 ± 0.96^{abc}	43.71 ± 2.00^{e}	46.39 ± 3.54^{e}
M	66.78 ± 2.64^{b}	89.79 ± 3.00^{cd}	99.23 ± 1.15^{de}	60.78 ± 1.29^{b}	70.04 ± 1.18^{d}	68.73 ± 2.63^{d}	28.12 ± 0.36^{bcd}	33.64 ± 0.99^{d}	31.82 ± 1.64^{cd}
E	52.72 ± 2.07^{a}	56.06 ± 2.03^{ab}	60.98 ± 1.69^{ab}	48.16 ± 1.92^{a}	53.63 ± 0.77^{ab}	54.61 ± 1.71^{ab}	22.23 ± 0.73^{a}	25.51 ± 0.67^{ab}	27.59 ± 0.57^{abc}
European larch (*Larix decidua*)		**							
A	59.96 ± 6.67^{def}	73.55 ± 4.11^{f}	70.12 ± 5.62^{ef}	12.92 ± 1.00^{a}	26.90 ± 5.79^{b}	24.07 ± 0.82^{b}	14.42 ± 1.31^{cd}	16.84 ± 0.90^{de}	17.93 ± 0.75^{e}
M	11.49 ± 0.27^{a}	49.40 ± 0.82^{cd}	55.83 ± 1.53^{cde}	12.91 ± 2.18^{a}	14.48 ± 1.95^{a}	14.35 ± 0.83^{a}	12.34 ± 0.14^{bc}	13.13 ± 0.52^{bc}	14.85 ± 1.28^{cd}
E	32.32 ± 0.37^{b}	43.63 ± 0.38^{bc}	47.01 ± 1.99^{cd}	6.61 ± 1.28^{a}	7.49 ± 0.55^{a}	8.25 ± 0.43^{a}	7.85 ± 0.30^{a}	10.97 ± 0.09^{b}	11.50 ± 0.21^{b}

2.3.1. Total phenol content (TPC)

TPC determination was completed using the Folin-Ciocâlteu assay at 760 nm with gallic acid as the standard (Singleton and Rossi, 1965). The results were expressed in mg equivalents of gallic acid/g dry weight (mg GAE/g dw.).

2.3.2. FRAP antioxidant capacity

The FRAP antioxidant capacity was determined according to Benzie and Strain (1996) at 593 nm using ascorbic acid as a standard. Results were determined in mg equivalents of ascorbic acid/g dry weight (mg AAE/g dw.).

2.3.3. DPPH antioxidant capacity

The DPPH assay was implemented using a slightly modified version of the method of Sharma and Bhat (2009) as follows: 2090 µl unbuffered methanol was mixed with 900 µl 2×10^{-4} M methanolic DPPH solution and 10 µl extract. After 30 min reaction time at room temperature in the dark, the decrease in absorbance was measured at 515 nm. Results were determined as IC_{50} (50% inhibition concentration) values in µg extractives/ml assay (µg/ml) units, representing the amount of extractives which will react with 50% of the added $DPPH^{\bullet}$ radicals in the total volume of the assay (3 ml) under these conditions (Hofmann et al., 2015). The IC_{50} values of standard compound (rutin, trolox, (+)-catechin) were also determined.

2.4. Content of extractives

Aliquots of the extracts (200 µl) were evaporated to dryness at room temperature in an aluminium crucible and the remaining solids were weighed. The total extractive content was expressed as mg extractives/mL extract unit. Results were used to calculate the DPPH IC_{50} values.

2.5. Statistics

In order to compare the respective chemical parameters of the cone extracts, ANOVA analysis was run using Statistica 11 (StatSoft Inc., Tulsa, USA) software applying the Tukey HSD calculation method for a *post-hoc* test.

3. Results and discussion

As polyphenols are an important non-enzymatic antioxidant in plants, measurements were first run for TPC. As the next step, the use of the other antioxidant assays was involved as well as a combined evaluation of the results.

3.1. Evaluation of total phenol content

The proper choice of extraction conditions (e.g. solvent composition, extraction time, temperature, etc.) is necessary to compare the concentrations of polyphenolic antioxidants of the investigated cone samples. The aqueous mixtures of methanol, ethanol, acetone, and ethyl-acetate are the most commonly used solutions for polyphenol extraction from plant tissues. The efficiency of a given solution depends very much on the structure of the phenolic compounds as well (Do et al., 2014). Ethanolic mixtures are known to be excellent for polyphenol extraction, and the use of ethanol (despite its higher cost compared with methanol) is also justified concerning human consumption, health, and toxicology. Methanolic solutions are generally the best for the extraction of low molecular weight polyphenols, while aqueous acetone solutions are more ideal for dissolving higher molecular weight flavanols (e.g. condensed tannins) from plant tissues (Dai and Mumper, 2010).

Adequately long extraction times are needed to ensure conditions of mass transport of extractives between plant material and solvent; however, overlong extraction times may lead to a decrease of polyphenol content and antioxidant capacity (Co et al., 2012; Hofmann et al., 2015).

TPC determination results are detailed in Table 1. According to the results, the aqueous acetone mixture was the most efficient solvent for the extraction of polyphenols for most samples. The methanolic solvent did not yield significantly higher amounts of polyphenols compared with the acetonic mixture, except for the mature cones of eastern hemlock. Overall, the ethanolic solvent yielded the lowest amounts of polyphenolic antioxidants.

Twenty-minute extraction times resulted in significantly higher TPC compared with 10 min extraction times in most samples. Nevertheless, between the values of 20 and 30 min, the only a significant increase occurred in green cones of Atlas cedar and Norway spruce. Neither solvent composition nor extraction time had a significant effect on the TPC of the green cone extracts of black pine. No decrease of the TPC was indicated in either of the samples when the extraction time was increased from 20 to 30 min, which indicated that degradation was not potentially taking place during the 30 min period.

According to our results, the highest polyphenol contents were found in the green cones of all of the investigated taxa, while lowest polyphenol levels were determined in the opened cones. Polyphenol content of plant tissues is influenced by various environmental factors (e.g. amount of solar radiation, precipitation, soil composition, etc.) as well as by genetic factors, age, and maturity of the tissue. In the course of ageing, and with the advance of the vegetation period, the phenolic composition can change markedly. During the ripening of fruits there is a general decrease of the amount of phenolic acids while the amount of anthocyanins increases (Manach et al., 2004). According to Tálos-Nebehaj et al. (2017), seasonal (May-September) polyphenol changes in the leaves of Hungarian forest trees depend on the species and type of the phenolic compound. As of yet, data on the change of the polyphenolic and antioxidant content during the ripening process of coniferous cones have not been found in the scientific literature.

According to Table 1., eastern hemlock and Norway spruce contained the highest amount of polyphenolic antioxidants not only in their green cones (e. h.: 118.91 ± 2.97 mg GAE/g dw., N. sp.: 105.58 ± 7.92 mg GAE/g dw.), but also in their mature cones (e.h.: 78.05 ± 2.42, N. sp.: 64.64 ± 2.68 mg GAE/g), evaluated from the 30 min acetonic extracts. Surprisingly the opened cones of Norway spruce also showed high values (46.39 ± 3.54 mg GAE/g). The tannin content of *Pseudotsuga* and *Tsuga* spp. cones has already been reported (*Tsuga heterophylla*: 3.13 wt.%, *Pseudotsuga menziensii*: 2.00 wt.%); however, the authors did not document either the phenophase of cone maturity or the month of the sample collection (Hernes and Hedges, 2004). The authors of the mentioned study found that the bark and green needles were generally a richer source of tannins compared with cones, yet their study did not investigate the amount of other types of polyphenolic compounds. Despite the high TPC of the Norway spruce cones, in the case of spruce by-products, only results for the bark can be found in the literature (Lazar et al., 2016; Ghitescu et al., 2015; Tanase et al., 2019), ranging from 37.3 to 43.1 mg GAE/g bark, which is slightly lower compared with the TPC the present study found for opened cones.

The total amount of extracted polyphenols itself is insufficient to determine the total amount of antioxidants in plant tissues as other types of antioxidants can also be present. On the other hand, the Folin-Ciocâltau assay (Singleton and Rossi, 1965), is limited in terms of specificity also interfering with other compounds as well (Prior et al., 2005).

In fact, each of the > 100 different assays currently known and used (Cornelli, 2009) for the measurement of antioxidant capacity measurement and radical scavenging ability is differently sensitive to different types of compounds, and none of the assays is individually able to measure the total antioxidant power of all compounds present in an extract. Because of the complexity of the samples, the use of multiple assays is recommended to assess the “overall” antioxidant potential of

Table 2
TPC[1], FRAP[2] and DPPH[3] antioxidant capacity of the cones (mean ± standard deviation). Different superscript letters indicate significant differences at $p < 0.001$ (FRAP, TPC) and at $p < 0.05$ (DPPH) between the samples with the 10 best values within a method.

	TPC (mg GAE/g dw.)			FRAP (mg AAE/g dw.)			DPPH (IC_{50}) (µg extractives/ml)		
	Green	Mature	Opened	Green	Mature	Opened	Green	Mature	Opened
Douglas fir	48.67 ± 0.90^{b}	17.24 ± 0.89	11.16 ± 0.66	23.36 ± 0.17^{b}	7.51 ± 0.28	3.61 ± 0.14	11.95 ± 0.79^{bcd}	14.40 ± 1.24^{d}	10.18 ± 0.79^{ab}
Eastern hemlock	118.91 ± 2.97^{d}	78.05 ± 2.57^{c}	3.31 ± 0.08	57.01 ± 3.13^{f}	38.31 ± 1.60^{cde}	0.98 ± 0.20	14.77 ± 2.85	9.84 ± 1.81^{ab}	25.89 ± 4.59
Black pine	47.17 ± 9.74^{b}	10.63 ± 0.36	9.20 ± 0.34	31.89 ± 1.31^{bcd}	6.24 ± 0.38	4.03 ± 0.27	14.81 ± 2.61	40.63 ± 0.86	17.02 ± 0.38
Atlas cedar	44.62 ± 0.16^{ab}	12.29 ± 1.20	6.05 ± 0.51	24.19 ± 0.45^{bc}	5.82 ± 0.24	2.53 ± 0.12	14.91 ± 2.00	25.44 ± 0.67	35.55 ± 1.55
Norway spruce	105.58 ± 7.92^{d}	64.64 ± 2.68^{bc}	46.39 ± 3.54^{b}	72.02 ± 8.76^{g}	50.19 ± 2.08^{ef}	28.35 ± 3.37^{bcd}	10.75 ± 0.32^{abc}	9.38 ± 1.14^{ab}	8.57 ± 0.17^{a}
European larch	70.12 ± 5.62^{c}	24.07 ± 0.82^{a}	17.93 ± 0.75	40.39 ± 0.73^{de}	7.79 ± 0.52	8.07 ± 0.46^{a}	13.73 ± 1.30^{cd}	12.27 ± 1.14^{bcd}	14.39 ± 0.75^{d}

1: Total phenol content.
2: Ferric reducing ability of plasma.
3: 2,2-diphenyl-1-picrylhydrazyl.

complex plant extracts (Ghiselli et al., 2000).

3.2. Evaluation of FRAP and DPPH antioxidant capacity

As ethanolic solutions yielded the lowest TPC, these extracts were excluded from further evaluations. The FRAP and the DPPH assays were run accordingly for all acetonic and methanolic samples, but involved only the samples with 10 and 30 min extraction times (data not shown) to obtain detailed information on the antioxidant properties of the samples. It was concluded that best results were achieved for both the FRAP and the DPPH methods when using the acetone:water 80:20 v/v solution for 30 min, just as in the case of the TPC determination. Consequently, further discussion is limited only to the evaluation of these extract results. The TPC, FRAP, and DPPH extract results gained by applying optimum solvent composition and extraction time settings are summarized in Table 2.

The values determined with the FRAP assay basically followed the tendencies of the TPC: highest FRAP antioxidant capacity was determined in green cones, followed by mature, and opened cones, with the green cones of Norway spruce (72.02 ± 8.76 mg AAE/g dw.) and eastern hemlock (57.01 ± 3.13 mg AAE/g dw.) showing the overall highest values. Similar to the TPC determinations, the opened cones of Norway spruce had excellent FRAP levels (28.35 ± 3.37 mg AAE/g dw.). According to Lesjak et al. (2011, 2014), the FRAP of *Juniperus* spp. cones are quite variable, ranging from 3.61 ± 0.03 mg AAE/g dw. (*Juniperus macrocarpa* Sibth. et Sm.) to 35.26 ± 1.12 mg AAE/g dw. (*Juniperus sibirica* Burgsdorf.). Other study results on the FRAP antioxidant power of cone extracts of other conifers (*Cupressus sempervirens* L. and *Pinus pinaster* Ait.) are not comparable to the present results, as values were given in various units (Tumen et al., 2012; Tümen et al., 2018). Compared with other forestry by-products, the FRAP of the bark extracts (18.3 mg AAE/g dw. – 80.1 mg AAE/g dw. according to Hofmann et al., 2015) as well of the leaf extracts (11.59 ± 0.72 mg AAE/g dw. – 106.24 ± 3.10 mg AAE/g dw. according to Tálos-Nebehaj et al., 2017) of Hungarian forest trees is at about the same level as found for the cones in the present study.

The DPPH radical scavenging activity was characterized by the IC_{50} value (50% inhibition concentration), with low IC_{50} indicating high antioxidant capacities. Interestingly, the DPPH antioxidant power between different maturation stages did not show the same tendency for all of the investigated taxa. Usually the strongest DPPH activity was determined in green cones; however, for black pine, Douglas fir, European larch, and Norway spruce, the DPPH activity of opened cones was not significantly different or comparable with respective green cone values. Further analysis is needed to evaluate which compounds may be responsible for the high DPPH antioxidant activity found in the opened cones. Overall, the best results were found for Norway spruce (green: 10.75 ± 0.32 µg/ml, mature: 9.38 ± 1.14 µg/ml, opened: 8.57 ± 0.17 µg/ml) and the green cones of eastern hemlock (9.84 ± 1.81 µg/ml). Comparing with the IC_{50} values of standard compounds (trolox:4.29 µg/ml, (+)-catechin: 7.40 µg/ml, rutin: 13.94 µg/ml) determined with the same assay (Hofmann et al., 2015), the best-performing Norway spruce cone extract solutions have a DPPH activity similar to that of (+)-catechin.

Results were comparable with the DPPH IC_{50} values of wood bark extracts (e.g., *Fagus sylvatica*: 11.12 µg/ml, *Calocedrus formosana*: 23.0 µg/ml, *Quercus* spp.: 33.7 µg/ml, *Juniperus oxycedrus*:1.1 µg/ml and 10.3 µg/ml for methanolic and aqueous extracts respectively) (Hofmann et al., 2015; Wang et al., 2004; Fazli et al., 2013; Chaouche et al., 2015) and with those of leaf extracts of Hungarian forest trees (4.63 ± 0.88 µg/ml – 42.38 ± 0.88 µg/ml) (Tálos-Nebehaj et al., 2017).

3.3. Combined evaluation of antioxidant capacity

According to Table 2, TPC and FRAP level results followed the same

Table 3
Scores detremined for the TPC[1], FRAP[2], and DPPH[3] IC_{50} antioxidant capacities and the sum of scores for each sample representing the combined antioxidant values.

	TPC (score)			FRAP (score)			DPPH IC_{50} (score)			Sum of scores		
	Green	Mature	Opened	Green	Mature	Opened	Green	Mature	Opened	Green	Mature	Opened
Douglas fir	0.39	0.12	0.07	0.32	0.09	0.04	0.89	0.82	0.95	1.60	1.03	1.05
Eastern hemlock	1.00	0.65	0.00	0.79	0.53	0.00	0.81	0.96	0.46	2.60	2.13	0.46
Black pine	0.38	0.06	0.05	0.44	0.07	0.04	0.81	0.00	0.74	1.62	0.14	0.83
Atlas cedar	0.36	0.08	0.02	0.33	0.07	0.02	0.80	0.47	0.16	1.49	0.62	0.20
Norway spruce	0.88	0.53	0.37	1.00	0.69	0.39	0.93	0.97	1.00	2.82	2.20	1.76
European larch	0.58	0.18	0.13	0.55	0.10	0.10	0.84	0.88	0.82	1.97	1.16	1.04

1: Total phenol content.
2: Ferric reducing ability of plasma.
3: 2,2-diphenyl-1-picrylhydrazyl.

tendencies, while the DPPH IC_{50} values showed a different trend between samples, which can possibly be explained by the diverse selectivity of methods to different compounds (Apak et al., 2007; Müller et al., 2011). In order to obtain a comprehensive measure of the overall antioxidant efficiency of the cone extracts and to take into respect the different selectivity of methods, the summarized evaluation of results of the different methods were carried out.

This was achieved in the present work by a scoring system described in detail in Tálos-Nebehaj et al. (2017): In the case of TPC and FRAP assays, 0 points were assigned to the weakest values and 1 to the best values within each assay, using linear approximation for the in-between values. For the DPPH values, opposite scoring was used as the lowest IC_{50} value (score: 1) represented the highest antioxidant capacity, and the highest IC_{50} (score: 0) indicated the weakest antioxidant power. The TPC, FRAP, and DPPH scores were summarized sample-wise to assess the measure of the overall antioxidant efficiency. Results of the evaluation are presented in Table 3.

Highest scores were determined for green cones, followed by ripe, and opened cones. The only exceptions were black pine where the score of mature cones (0.14) was lower compared with opened cones (0.83), and Douglas fir where the difference between ripe (1.03) and opened cones (1.05) is negligible.

The overall highest scores were determined in green cones of Norway spruce (2.82) and eastern hemlock (2.60). Comparing the mature and opened cones, which are most likely to be harvested and collected in forestry practice, the best values were once again found in the ripe cones of Norway spruce (2.20) and eastern hemlock (2.13), and for the opened cones of Norway spruce (1.76), Douglas fir (1.05), and European larch (1.04).

The applied evaluation method was suitable for the complex assessment of the antioxidant properties of coniferous cone extracts. As to the best of our knowledge such method has not been presented yet in scientific literature for cone extracts. It was also the first time that complex antioxidant properties and total polyphenol content data has been published for the investigated species, also focusing on the comparison of cone composition at different ripening stages.

Considering possibilities of future uses of antioxidants from conifer cones, Norway spruce might be of special interest, as it is one of the most widespread coniferous tree species in Europe with significant ecological, industrial, and economic importance (Meloni et al., 2007; Lamedica et al., 2011). According to the present results, Norway spruce cones possess the highest antioxidant contents. In this respect, the future evaluations of other bioactive properties related to antioxidant capacity including anti-viral, anti-bacterial, anti-proliferative, and other properties, have to be investigated.

4. Conclusions

Our study concludes that ultrasonic extraction for 30 min using acetone:water 80:20 v/v resulted in the highest yield of polyphenolic antioxidants. Accordingly, we will apply this solvent composition in future investigations. Comparing the total polyphenol contents of the cones of the arbitrarily selected taxa of the present study showed that green cones contained the highest amount of polyphenols followed by ripe cones and opened cones. Overall, Norway spruce and eastern hemlock contained the highest levels of polyphenols, not only in their green cones, but also in their mature cones. The FRAP antioxidant capacity of the samples followed the same tendencies as established for the total phenol content, while results of the DPPH assay showed different results, presumably because of the different selectivity of the method. Antioxidant assay results were combined by introducing a scoring system to determine and compare the overall antioxidant power of the samples. Green cones showed the overall highest antioxidant power for each taxon. The best performing samples were the green and ripe cones of Norway spruce and eastern hemlock. Surprisingly the opened cones of Norway spruce also received high scores. Results provide methodology or the future systematic evaluation and comparison of the antioxidant properties of cones of other taxa. The evaluation of the anti-viral, anti-bacterial, and anti-proliferative properties of the samples with the highest scores is also essential to track possible bioactive effects. Future separation and identification of the compounds responsible for antioxidant and possible bioactive effects is also important, not only to find potential bioactive compounds responsible for beneficial effects, but also to contribute to the identification of the polyphenolic compounds found in coniferous cones.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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