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RESEARCH ARTICLE

Polyphenols composition, antioxidant and antimicrobial properties of *Pinus sylvestris* L. shoots extracts depending on different drying methods

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ABSTRACT

Scots pine (*Pinus sylvestris L.*) shoots have been increasingly commonly used as functional food or its component, the shoots contain various active components, unknown from other raw materials. The objective of the study was to assess the influence of the drying method on the content of bioactive compounds and antioxidative and antimicrobial activity of pine shoots. It was demonstrated that freeze drying (PSL), vacuum drying (PSP) and natural drying (PSN) have significant impact on the physicochemical properties, content of bioactive compounds and antioxidative activity of the prepared ethanol-water extracts. The content of the studied compounds varied significantly in the tested shoots. In spectrophotometric testing the highest total flavonoid content was demonstrated in the PSP sample, at 5.51 mg quercetin/g dw. On the other hand, the reducing capacity was as follows: PSN > PSP > PSL in the range from 13.4 to 5.73 mg gallic acid/g dw. However in assay conducted using HPLC methods the highest content of polyphenols characterized extract from freeze-dried raw material (9151.15 μg/g), followed by vacuum-dried (8264.57 μg/g), and the lowest content of phenolic compounds was found in convection-dried shoots (7621.76 μg/g). The studied extracts demonstrated antioxidative properties, both in ferric reducing antioxidant power assay (FRAP) as well as in free radical quenching measurement (DPPH). All of the studied extracts demonstrated antimicrobial and fungicidal properties, and they were particularly efficient in the case of gram-negative bacteria.

Keywords: Antioxidant properties; Drying conditions; Microbial growth; *Pinus sylvestris* L.; Polyphenols

INTRODUCTION

Researchers are currently looking natural sources of health-promoting compounds that could be used in food. The growing interest in food products characterized by high antioxidative potential results from the efficiency of these compounds in prevention of diseases associated with systemic oxidative stress, i.e. cardiovascular diseases, tumors, neurodegenerative disorders and metabolic disturbances (Roleira et al., 2015). As a result of new reports on the role of antioxidants, products rich in these compounds (especially of vegetable origin) have become increasingly desirable on the market, although numerous cultures have traditionally used vegetable products for hundreds and even thousands of years to treat various diseases, as well as to support health, stemming from the traditional use, abundant availability and low price, as well as the belief in the efficiency of these preparations (Ramana et al., 2018). Obtaining extracts at a large scale requires use of various drying methods in order to fix raw materials and retain their bioactive properties. Traditional and thus natural and the least expensive raw material drying methods include sun drying and air drying. Dehydration under the impact of these factors, due to the prolonged duration, possible contaminants and limited efficiency may not be used for numerous raw materials (Villalobos et al., 2016). Modern drying methods that are gaining popularity include, among others, microwave drying, vacuum drying, freeze drying, which may help enable retaining high quality of considerable amount of bioactive compounds, while optimizing parameters significant in economic terms. These methods largely enable minimizing surface overheating, and reduce the drying time.

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Scots pine is a long-lived coniferous tree of high economic significance, whose shoots and leaves are characterized by high content of bioactive compounds. These trees comprise 68% of Polish forests and have been traditionally used to produce liqueurs, ointments and syrups considered to have therapeutic properties (Bączek et al, 2017). Extracts from bark of *Pinus sylvestris* showed antioxidant activity in AAPH and DPPH assays as well as reducing and metal chelating properties (Nazari et al., 2013; Sokół-Łętowska et al., 2007). It has been demonstrated that the shoots and leaves of these trees contain, among others, essential oils rich in monoterpenes, i.e. alpha-pinene, beta-pinene, delta-3-carene, beta-myrcene, limonene, p-cymene, exhibiting strong antibacterial, antifungal, antiviral and antioxidative properties (Ács et al., 2018). Currently, the majority of researchers focus on the use of isolated essential oils, although the extracts alsho exhibit therapeutic potential, for example as chemopreventive or chemotherapeutic agent (Hoai et al., 2015; Nicolato et al., 2009). Recently there were attempts to use pine's components in food fortification, fore example in kefir and beer. Such products show higher antioxidant capacity, better storage stability and can positively influence quality of product (Penkina et al., 2017; Semeniuc et al., 2016). Currentliterature contains data on the use of shoots in food technology, as well as in pharmacy, yet the mode in which drying would influence the products' activity has not yet been studied. The objective of the study was to determine antioxidative and antimicrobial properties of *Pinus Sylvestris* L. shoots and impact of different drying methods on physicochemical, antimicrobial and antioxidative properties of Scots pine shoot extracts. Therefore, in the present study the shoots were dried via freeze drying, natural-air drying and vacuum drying and evaluated by physical, chemical and microbiological assays.

MATERIALS AND METHODS

Material

The study material consisted of *Pinus sylvestris* L. shoots collected in 2019 from the arboretum in Zielonka (Poland, 17°06'33"E, 52°06'33"N), a part of the Forest Experimental Department of Poznan University of Life Sciences. The shoots were collected in August and fixed using three different drying methods: (1) freeze drying (PSL) for 48h in an Alpha 1-2 LSC freeze drier (Christ, Germany); (2) vacuum drying (PSP) at 60 °C under the pressure of 470 mbar for 48h in a VO29 drier (Memmert, Germany); (3) natural-air drying (PSN) at 21 °C for 72h. The dried needles were crushed in a Grindomix GM 200 by Retsch (Haan, Germany) for 15 seconds at a rate of 500 rpm at 21 °C to a particle size of 0.5 - 0.9 mm.

Extraction

Ethanol-water extract (40%) was obtained by mixing 5 g of raw material with 150 mL of solvent (Sigma-Aldrich, Germany). The samples were shaken in a water bath for 15 min at 21 ◦C at constant amplitude. The extract was decanted and filtered using Whatman No. 4 paper three times. Obtained supernatants were stored at -21 °C for no more than two weeks before further analyses. Each measurements and analysis for each extract were conducted in triplicate.

Density and extraction yield

Extractable yield was measured and calculated according to methodology by Pham et al (Pham et al., 2015) and expressed as % dried extract. Density was measured by pipetting 1 ml of extract with automatic single channel pipette (Thermo Fisher Scientific, USA) on weighing vessel and weighed using laboratory scale, expressed in g/ml.

Instrumental analysis of color

The color of the extracts (reflectance values: L*, a* and b*) was measured using CM-5 spectrometer (Konica Minolta, Japan) according to methodology described by the device producer. L*, a*, b* values were determined using Illuminant D65 and an observer angle of 10°, color temperature equaled 6504 K.

Ascorbic acid content

Vitamin C (ascorbic acid) content was determined according to the modified method described by Ajila et al. (Ajila et al., 2007). The absorbance drop was calculated. Ascorbic acid was used as a standard. The calibration curve was performed using AA standard solutions. The linearity of the curve coefficient – r^2 equaled 0.98. The final results are given in mg AA/g dw.

Folin–Ciocalteu reagent assay

Reducing capacity was determined using Folin-Ciocalteu reagent (FCR) of the obtained extracts was determined using the method of Kobus-Cisowska et al. with minor modifications (Kobus-Cisowska et al., 2020). The reducing capacity was expressed as mg of gallic acid (Sigma-Aldrich, Germany) equivalents (GAE) per 1 g (mg/1 g) of dry mass using the calibration curves of gallic acid.

Total flavonoid content

The total flavonoid content was determined using the procedure described by Meda et al. (Meda et al., 2005). The total flavonoid content was determined using a standard curve with quercetin (Sigma–Aldrich, Germany) as the standard. The mean of three readings was used and expressed as mg of quercetin equivalents QE/1 g of raw material.

Content of flavonols and phenolic acids

The procedure was based on the method published by Kobus et al. (Kobus-Cisowska et al., 2019b).

Content of chlorophylls and carotenoids

The carotenoids and chlorophyll were determined using the standard method (Kobus-Cisowska et al., 2019b) and expressed in mg/g of dry product.

Ferric reducing/antioxidant power assay

The antioxidant properties of the water-ethanol extracts were determined using a ferric reducing/antioxidant power assay (FRAP method) according to procedure described by O'Sullivan et al. (O'Sullivan et al., 2013). A calibration curve was constructed using $FeSO_4$ $7H_2O$. Samples were incubated for 30 min and the absorbance was measured at 593 nm (Metertech SP880, Taiwan). Data were expressed as μM $FeSO_4$/g dry mass.

DPPH radical scavenging activity

The inhibition capacity of DPPH was investigated according to the procedure performed by Szczepaniak et al. (Szczepaniak et al., 2019). A calibration curve was prepared using a Trolox standard solution. The decrease in DPPH absorbance (A) was measured at 517 nm according to the blank (A'). The inhibition capacity of the DPPH radical was calculated and expressed as a percentage.

Antimicrobial activity

Antimicrobial activity of extracts was measured according to Kobus-Cisowska et al. as the inhibition of the growth of the indicator microorganisms: *Klebsiella pneumoniae* ATCC 31488, *Salmonella enteritidis* ATCC 13076, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* ATCC 19606, *Enterococcus faecium* ATCC 27270, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* ATCC 25923, *Lactobacillus fermentum* ATCC 14932, *Clostridium butyricum* ATCC 860, *Listeria monocytogenes* ATCC 19115, *Bacillus coagulans,* GBI-30, 6086, *Candida utillis* ATCC 9950, *Aspergillus* sp., *Fusarium* sp. (Kobus-Cisowska et al., 2019b). Bacteriostatic properties were determined by measuring the diameter of the growth inhibition zone (indicator strain growth limitation).

Statistical analysis

All assays were conducted in triplicates and results expressed as mean ± SD. One way ANOVA testing was used to analyze statistical differences amongst the various extracts for phenolic compound contents and different antioxidant assays with least significant difference (LSD). The P value less than 0.05 was assumed as a level of significance. Correlations between the content of components and antioxidant attributes were determined by Pearson's correlation coefficients. Additionally, the analysis of the principal components was used (PCA). Statistical analyses were calculated using Statistica 13.3 software (Statsoft, Poland).

RESULTS

Extraction yield and physical properties of extracts

Extraction yield, density, pH and extract color were examined and are presented in Table 1. The yield level was as follows: PSL>PSP>PSN, these values correlated with pH, but did not correlate with extract density. In the case of material dried using freeze drying, high yield of 32.05% was obtained, for the natural-air-dried raw material in room temperature, a 10-fold lower yield was obtained. The density remained at a similar level between all samples, with the highest density (0.973 g/ml) characterizing PSL sample, whereas lowest PSP (0.932). The indicative of hydrogen ion concentration was similar for PSL and PSP samples (4.07 and 4.15, respectively), indicating their acidity, whereas higher for PSN, demonstrating its more alkaline pH. The extracts differed significantly in terms of color measured in the CIELab space. The PSL sample had the highest lightness value (L^* = 75.01), whereas PSN was the darkest sample (L^* = 62.03). The a^* and b^* values indicating colors in the range from yellow and blue, also differed significantly between the samples, showing the impact of drying on the content of pigments in the extracts.

Content of ascorbic acid, flavonoids, chlorophylls and carotenoids

The content of the studied compounds varied significantly in the tested shoots. The highest content of vitamin C characterized PSP sample (59.12 mg/g), where this value was close to 3-fold higher than in PSN sample (20.96 mg/g) and as much as 7.5 fold higher than in PSL sample (7.81 mg/g), although the difference was not statistically significant. The highest concentration of total chlorophyll and carotenoids was observed in the extract of air-dried

Table 1: Yield of *Pinus sylvestris* L. needles extraction.

Sample	pH	Density (g/ml)	Extract yield (%)	Color		
				*L**	*a**	*b**
PSN	5,48^a±0,00	0,961	3,21^a±0,01	62,03^a±0,05	5,76^a±0,11	61,68^a±0,15
PSL	4,07^b±0,02	0,973	32,05^b±6,66	75,01^b±0,01	1,08^b±0,01	26,66^b±0,36
PSP	4,15^c±0,00	0,932	20,84^c±1,60	70,32^c±0,27	2,22^c±0,05	57,41^c±0,03

Results are mean values of three determinations ± standard deviation. Values sharing the same letter in a column are not significantly different ($p \leq 0.05$).
PSN – air-dried needles of *Pinus sylvestris* L.; PSP – vacuum-dried needles of *Pinus sylvestris* L; PSL – freeze-dried needles of *Pinus sylvestris* L

shoots (PSN), respectively 126.11 mg/g dw and 49.34 mg/g dw, the lowest content of these compounds was exhibited by the extract from vacuum-dried leaves (PSP). The difference of total chlorophyll concentration was not statistically significant between PSN and PSP, and between PSP and PSL. In terms of total flavonoid content the values are analogous to the content of ascorbic acid, the highest content was demonstrated in the PSP sample, at 5.51 mg quercetin/g dw, in case of PSN and PSL samples the content of flavonoids was similar and amounted to 4.44 and 4.22 mg quercetin/g dw and the difference between these samples was not statistically significant. These values show that drying has a significant impact on the content of individual compounds in the raw material.

Content of flavonols and phenolic acids by HPLC

The content of polyphenols and phenolic acids depended on the applied drying method. The highest content of polyphenols characterized extract from freeze-dried raw material (9151.15 µg/g), followed by vacuum-dried (8264.57 µg/g), and the lowest content of phenolic compounds was found in convection-dried shoots (7621.76 µg/g). The most abundant phenolic compound found in the extracts was naringenin (3221.25 – 3868.04 µg/g), ferrulic (1222.65- 1380.69µg/g) and caffeic acid (954.89 - 1432.17 µg/g). In addition, high contents of hydroxybenzoic, chlorogenic, coumaric and vanillic acid have been identified in the extracts.

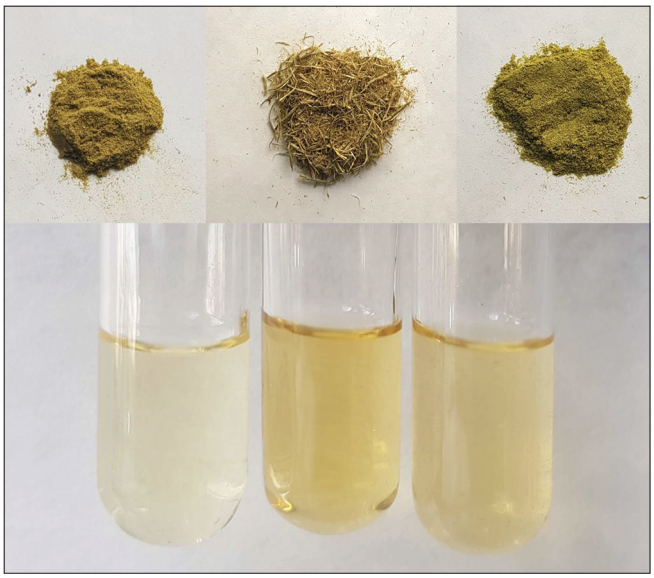

Fig 1. Ground dried shoots and of *Pinus sylvestris* L. and extracts. Order of samples from left: PSL, PSN, PSP

Antioxidative properties

The studied extracts demonstrated high antioxidative properties, both in ferric reducing antioxidant power assay (FRAP) as well as in free radical quenching measurement (DPPH) and Folin-Cicoalteau reagent assay (FCR). In the case of Fe^{3+} reduction to $Fe^{2+,}$ the most pronounced activity was shown by the PSP extract (47.25 µM FeSO4/g dw), but was not statistically different than PSN (37.79 µM $FeSO_4$/g dw), the smallest activity was pronounced by PSL (21.79 µM FeSO4/g dw), but the difference was not significant compared to PSN. Free radical quenching capacity DPPH was expressed in % of quenching, but also in the form of Trolox equivalent. The assessment demonstrated that all samples are characterized by the capacity to scavenge free radicals to a similar level, with the strongest properties found for the PSL sample (66.7% and 339 µM Trolox/g dw), and PSN (64.90% and 332.25 µM Trolox/g dw), statistically different was PSP sample (55.92% and 299.72 µM Trolox/g dw). On the other hand, according tothe FCR assay reducing capacity was as follows: PSN>PSP>PSL in the range from 13.4 to 5.73 mg gallic acid/g dw.

Antimicrobial activity

All of the studied extracts demonstrated antimicrobial and fungicidal properties, and they were particularly efficient in the case of gram-negative bacteria. The results were variable, however the PSL extract was characterized by the strongest biocidal properties towards the majority of microorganisms.

PCA projection (biplot) of results for qualitative analysis of *Pinus sylvestris* L. leaf extracts in set of two first components (PC1 and PC2), responsible for approximately 58% composition deviation, presented heterogeneity of tested

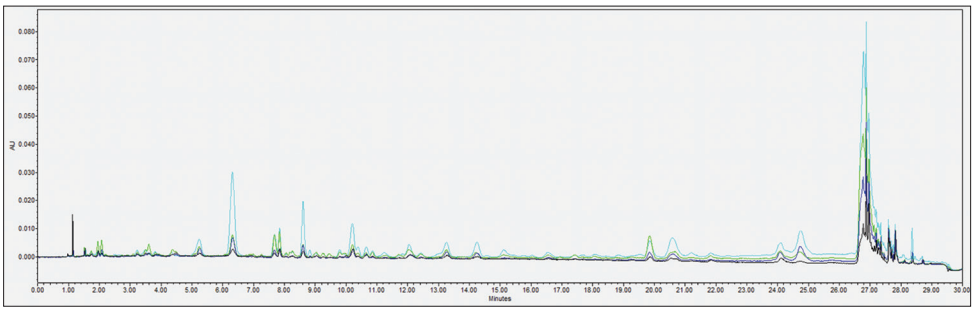

Fig 2. HPLC chromatogram of acids and standards. Legend: PSL – black line, PSP – blue line, PSN – green line, standards - light blue line

samples in terms of bioactive ingredients composition and the degree of influence of selected compounds on extracts' characteristic (Figure 3). The position of extracts on the right part of the figure indicates high deviation between samples in terms of the content of analyzed compounds and assayed antioxidative activity.

DISCUSSION

Considering the continuing growth of interest for vegetable products with health-promoting properties, it is necessary to evaluate these raw materials in order to provide consumers with functional products having the desired, efficient action (Griffiths et al., 2016). The main compounds with antioxidative nature in plants include phenolic compounds, carotenoids, chlorophylls and vitamins, and thus in vitro determination of the content of these compounds is the basic method of evaluation for the potential antioxidative properties of products (Bungau et al., 2019). Considering that determination of each component individually in complex systems such as food products would be impossible to carry out, methods such as FRA assay, FCR assay, DPPH scavenging are used, allowing a rapid, simple and sensitive determinations. Thus, these methods have been applied in the present study (Benzie and Choi, 2014).

The conducted study demonstrated that the method and conditions of the applied raw material drying have considerable impact on physical properties and antioxidant content, as well as antioxidative properties of an extract. Drying method impact on extraction yield and content of bioactive compounds is well documented regarding many raw materials as a consequence of the matrix changes during the drying process (Górnaś et al., 2014; Youssef and Mokhtar, 2014). Low efficiency characterized the air-dried shoots, whereas vacuum-dried and freeze-dried shoots were characterized by considerably higher extraction yields. This may be caused by the specificity of pine shoots containing waxes, resins and essential oils, differing from the majority of plant materials. Similar relationship was demonstrated in the study of Pham et al., where drying at 30 °C and 25 °C resulted in very low extraction efficiency, which was most likely linked to the differences in the permeability and solubility of the dried material (Pham et al., 2015). The authors noted significant differences in the color, indicating considerable impact of drying on the content of bioactive compounds, in particular of pigments (Pham et al., 2015). Spectrophotmetric testing demonstrated that pine shoots can be a source of vitamin C, which is a strong antioxidant, and fulfills a wide range of biological functions. The content of ascorbic acid differed significantly depending on the drying method. Literature includes reports on vitamin C degradation as a result of drying is frequently observed due to its susceptibility to numerous factors, i.e. light, temperature, humidity (Deng et al., 2018). In addition, vitamin C content in pine shoots, varies depending on the season of the year or the pollution level (Kalugina et al., 2018). Temperature and relative humidity are among the most important factors influencing the change of coloration and thus pigment degradation (Shahabi et al., 2014). The relationship between temperature growth and decrease of chlorophyll

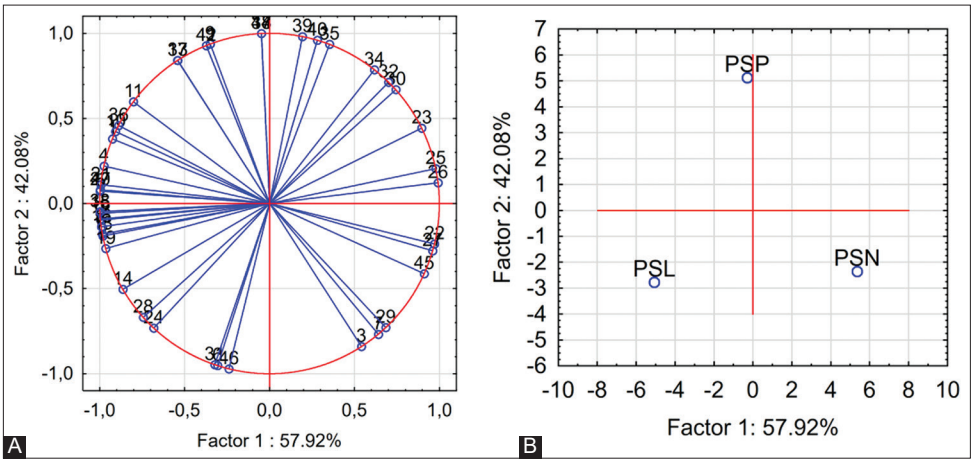

Fig 3. Principal component scatter diagram for tested samples (a) and principal components projection (b).Variables numbers as follows: 1. Gallic acid; 2. 2,5.dihydroxybenzoic acid; 3. 4.hydroxybenzoic acid; 4. caffeic acid; 5. syringic acid; 6. p.coumaric acid; 7. ferulic acid; 8. chlorogenic acid; 9. sinapic acid; 10. t.cinnamic acid; 11. vanilic acid; 12. salicylic acid; 13. Naringenin; 14. Vitexin; 15; Rutin 16. Quercitin; 17. Apigenin; 18. Kaempferol; 19. Luteolin; 20. Color L*; 21. Color a*; 22. Color b*; 23. Ascorbic acid; 24. Chlorophyll a; 25. Chlorophyll b; 26. total carotenoids 27. total flavonoids; 28. total polyphenol; 29. DPPH; 30. FRAP; 31. Klebsiella pneumoniae ATCC 31488; 32. Salmonella enteritidis ATCC 13076; 33. Pseudomonas aeruginosa ATCC 27853; 34. Acinetobacter baumannii ATCC 19606; 35. Enterococcus faecium ATCC 27270; 36. Staphylococcus aureus ATCC 25923; 37. Lactobacillus fermentum ATCC 14932; 38. Clostridium butyricum ATCC 860; 39. Listeria monocytogenes ATCC 19115; 40. Bacillus coagulans; 41. Candida utillis ATCC 9950; 42. Aspergillus sp.; 43. Fusarium sp.; 44. pH; 45. Density; 46. extraction yield.

Table 2: The content of chlorophyll and carotenoids in *Pinus sylvestris* L.

Extract	Ascorbic acid (mg/g dw)	Chlorophyll A (mg/g dw)	Chlorophyll B(mg/g dw)	Total Chlorophyll (mg/g dw)	Carotenoids (mg/g dw)	Total flavonoids (mg quercetin/g dw)
PSN	20,96^{a}±8,46	43,30^{a}±9,96	71,66^{a} ±9,04	126,11^{a}±12,49	49,34^{a}±2,07	4,44^{a}±0,34
PSP	59,12^{b}±12,25	13,43^{a}±4,44	11,51^{a}±2,24	23,05^{ab}±3,15	17,72^{b}±2,74	5,51^{b}±0,52
PSL	7,81^{a}±1,77	32,53^{b}±9,31	45,48^{a}±9,58	68,95^{b}±7,78	24,18^{c}±4,75	4,22^{a}±0,64

The mean values in the column marked with different small letters indicate the significance of differences ($p \leq 0.05$). dw - dried weight of raw material

Table 3 : HPLC analysis of phenolic compounds in *Pinus sylvestris L.*

Compound (µg/g dw)	PSN	PSP	PSL
gallic acid	134,57^{a}±2,38	174,12^{b}±5,22	184,54^{b}±8,58
2,5-dihydroxybenzoic acid	10,65^{a}±0,59	13,12^{b}±0,97	18,66^{c}±0,73
4-hydroxybenzoic acid	674,09^{a}±2,27	619,94^{b}±1,57	671,68^{a}±29,67
caffeic acid	954,89^{a}±0,60	1336,18^{b}±31,73	1432,17^{c}±66,86
syringic acid	92,11^{a}±2,07	108,76^{b}±0,55	117,21^{c}±3,63
p-coumaric acid	234,89^{a}±0,99	220,61^{a}±3,08	256,45^{b}±14,92
ferulic acid	1380,69^{a}±1,9	1222,65^{b}±4,09	1330,81^{c}±37,18
chlorogenic acid	311,71^{a}±2,15	347,65^{b}±1,88	418,68^{c}±8,57
sinapic acid	36,95^{a}±0,06	62,59^{b}±4,03	42,92^{c}±1,4
t-cinnamic acid	72,35^{a}±1,43	114,50^{b}±2,97	120,49^{b}±1,38
vanilic acid	198,55^{a}±0,65	223,36^{b}±0,42	222,14^{b}±9,85
salicylic acid	6,50^{a}±0,44	13,04^{b}±0,88	21,47^{c}±0,53
naringenin	3221,25^{a}±5,87	3456,63^{b}±5,01	3868,04^{c}±79,99
vitexin	18,92^{a}±1,36	24,13^{b}±3,83	56,97^{c}±2,28
rutin	103,16^{a}±4,21	111,90^{b}±4,33	124,80^{c}±4,55
quercetin	155,76^{a}±7,49	187,68^{b}±10,98	243,74^{c}±7,4
apigenin	10,36^{a}±0,62	12,54^{b}±0,51	11,82^{b}±0,44
kaempferol	0,26^{a}±0,06	10,15^{b}±2,69	0,30^{a}±0,02
luteolin	4,12^{a}±0,25	5,02^{b}±0,43	8,26^{c}±0,22
Total phenolic compounds	7621,76^{a}±14,43	8264,57^{b}±48,09	9151,15^{c}±118,31

Results are mean values of three determinations ± standard deviation. Values sharing the same letter in a line are not significantly different ($p \leq 0.05$)

Table 4: Radical scavenging and antioxidant activity of *Pinus sylvestris* L. with DPPH and FRAP methods

Assay	PSN	PSP	PSL
DPPH scavenging effect (%)	64,90^{a}±2,9	55,92^{b}±4,41	66,76^{a}±5,42
DPPH (µM Trolox/g dw)	332,25^{a}±10,49	299,72^{b}±15,97	339,00^{a}±19,61
FRAP (µM FeSO4/g dw)	37,79^{a}±3,64	47,25^{ab}±14,06	21,79^{ac}±4,36
FCR (mg GAE /g dw)	13,4^{a}±4,07	8,34^{b}±2,01	5,73^{c}±2,55

Results are mean values of three determinations ± standard deviation. Values sharing the same letter in a line are not significantly different ($p \leq 0.05$)

content and carotenoids in plant material in the process drying has also been demonstrated (Oliveira et al., 2015). Similarly to this study, numerous other works showed that the freeze-drying method enables high pigment concentration to be retained. Feng et al., who studied lettuce cubes, demonstrated that freeze-drying allowed retention of higher amount of chlorophylls as compared with hot air drying and microwave drying, both in vacuum conditions as well as in bed drying (Feng et al., 2012). Freeze drying was also the most favorable method in the study of green tea, where this method enabled obtaining several-fold higher content than for fresh leaves, as well as sun dried and shade dried leaves (Roshanak et al., 2016). On the other hand, Barisa et al. demonstrated minor differences between the content of chlorophyll and carotenoids between samples dried with the following methods: convection oven drying, microwave drying, air drying with and without sun exposure and food dehydrator drying. In turn, Kumar et al. demonstrated that drying leaves of *Hibiscus sabdariffa* L. at room temperature was more favorable than freeze drying and other methods (Branisa et al., 2017; Kumar et al., 2015). Lower yields of these compounds in present study in PSL sample could be also attributed to characteristic structure of Pine shoots. As the outer layer is covered by epicuticular waxex, freeze-drying of whole shoots could contribute to build-up of internal pressure and lead up to material damage, making it more susceptible to external conditions (Bhatta et al., 2020). Therefore, it can be expected that raw material type also has a decisive impact on the stability of pigments during drying with different methods. Numerous studies determined that the content of flavonoids determines the antioxidative properties of extracts (Kobus-Cisowska et al., 2019a; Kulczyński et al., 2016; Szczepaniak et

Table 5. Antibacterial activity of *Pinus sylvestris L.*

Microrganism	Growth inhibition zone [mm]		
	PSN	PSP	PSL
Gram(-) bacteria			
Klebsiella pneumoniae ATCC 31488	22±2	12±2	25±1
Salmonella enteritidis ATCC 13076	16±2	18±7	20±3
Pseudomonas aeruginosa ATCC 27853	27±3	25±2	28±5
Acinetobacter baumannii ATCC 19606	20±2	18±2	23±3
Gram(+) bacteria			
Enterococcus faecium ATCC 27270	18±2	19±2	19±9
Staphylococcus aureus ATCC 25923	19±2	19±3	21±7
Lactobacillus fermentum ATCC 14932	13±2	15±3	14±8
Clostridium butyricum ATCC 860	17±2	16±2	20±4
Listeria monocytogenes ATCC 19115	19±2	18±2	21±5
Bacillus coagulans GBI-30, 6086	19±2	20±1	22±8
Fungi			
Candida utillis ATCC 9950	6±1	7±1	9±1
Aspergillus sp.	5±1	4±1	7±5
Fusarium sp.	5±1	5±1	6±4

al., 2019). The test of total flavonoids content showed that the extract obtained from freeze-dried shoots was characterized by lower content of these compounds Based on the example of numerous plant raw materials, it was demonstrated that drying at elevated temperature may lead to increased concentration of phenolic compounds, which is caused by altered activities of various key enzymes of phenolic biosynthetic pathways, increased activity of enzymes such as polyphenol oxidase, phenoloxidase cause abiotic stress and decreased water content in cells [39]. On the other hand, the content of 19 flavonoids and phenolic acids determined via HPLC exhibited highest concentration for the PSL and lowest for the PSN sample. In line with observations of numerous authors, pine needles were characterized by high quercetin content, whereas narginenin and ferrulic acid were predominant among phenolic acids (Metsämuuronen and Sirén, 2019). All samples were characterized by high antioxidative activity in the free radical DPPH assay and in ferric reducing antioxidant power assay. The order of activity was as follows: PSL>PSP>PSN for DPPH and PSP>PSN>PSL for FRAP. The results for FCR assay were slightly different, the sample with highest reducing capacity was PSN and PSL sample had the lowest reducing activity. These differences showed between different methods may stem from the different reactivity of the tested components in extracts towards the studied reagent. For example, FC reagent is nonspecific to phenolic compounds and can be reduced by many nonphenolic compounds such as vitamin C, which concentration was lowest in PSL sample (Górnaś et al., 2016). However, in the case of numerous phenolic compounds, relationship explained via bell-shaped curves has been observed, as many compounds can be efficient antioxidants at very low concentrations but rather inefficient or pro-oxidative depending on the concentration, conditions and co-occurrence of other compounds (Eren-Guzelgun et al., 2018). Pine shoots have wide antimicrobial and fungicidal action, which has also been demonstrated herein. Other authors refer to the essential oils contained in coniferous trees as compounds with highest antimicrobial potential, however some phenolic compounds also demonstrate this effect, such as quercetin, ferrulic acid and apigenin contained in Scots pine (Nazzaro et al., 2017).

CONCLUSIONS

Pine shoots form a raw material characterized by containing numerous bioactive compounds with high antioxidative, reducing and antimicrobial properties. This raw material may be applied in functional foods due to the wide spectrum of potential health promoting action, low price and good availability. However, the conducted study provide evidence that the applied fixing methods, i.e. drying leave marked impact on the physicochemical properties of the obtained extracts and further research is needed in order to optimize the process and to evaluate the applicability of pine shoots.

Authors' contributions

Conceptualization and methodology: Joanna Kobus-Cisowska; formal analysis: Marcin Dziedziński, Daria Szymanowska-Powałowska, Kinga Stuper-Szablewska; resources: Marlena Baranowska; writing—original draft preparation: Marcin Dziedziński; supervision: Joanna Kobus-Cisowska.

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Conflicts of Interest

The authors declare no conflict of interest.

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