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Theses of doctoral dissertation

Determination of antioxidant parameters in the leaf and bark tissues of Hungarian forest tree species

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Introduction

In the last decades the number of investigations in the field of polyphenolic antioxidant compounds has increased significantly. Especially the positive effects of these compounds on diseases have been highlighted, as nowadays the growing tendency of civilization diseases is noticeable. Although polyphenols have a significant antioxidant effect, not only these types of compounds are responsible for the antioxidant effects that can be found in plants, since the plant antioxidants can be varied. Moreover that is very important that the compounds with antioxidant properties in plants have other functions: carotenoids are present as pigments, terpenes act as allelopathic agents. The research has proven that the synthesis and accumulation of plant polyphenols is usually associated with biotic and abiotic stress and environmental responses at plant species. In addition, these compounds are not only antioxidants, but also they are antiviral, antimicrobial, and are also important in the human body. In the case of plant tissues, foods and other biological matrices, the reliable, reproducible and numerical determination of antioxidant properties is an important challenge. However, there's little result of these compounds from indigenous forest tree's tissues. In the case of forest trees, the investigation of antioxidant properties was mainly due to the examination of protective reactions (fungi or bacterial infection, climatic adaption, etc.) as well as the extracted and potentially usable materials from wood by-products (branches bark etc.). Although the research topic is significant by several aspects, similar investigations of Hungarian forest tree species have not yet been conducted.

The aims of the dissertation

The primary objective of the research was to compare the antioxidant properties of the extracts from the leaf and bark of the most important forest tree species in Hungary and the qualitative and quantitative determination of polyphenols responsible for antioxidant activity. The author found it important to determine in which extracts and under what conditions the maximum values can be measured, so with the help of this, later the industrial utilization of extracts can be considered.

1. As a first step, sampling, sample preparation and extraction procedures were optimized.
2. The aim was also to compare the antioxidant parameters of leaf samples from selected forest species at different points of the vegetation period to determine when they have the highest antioxidant capacity in the leaves. Tree species were selected to have one of their literal examples of antioxidant capacity, and also what has not been studied in this respect.
3. Another goal was to investigate the extracts of inner and outer bark of industrially important Hungarian forest trees. In the course of measurements the antioxidant capacity values and the total phenol contents were determined.
4. Finally, from selected forest tree species (European beech bark, European hornbeam leaf) the qualitative and quantitative determination of polyphenols has been carried out using HPLC-PDA-MS/MS technique.

Materials and Methods

1. Sampling and sample pretreatments

Collection of *leaf samples* was carried out at the Sopron University's Live Plant Collection. In October 2013, 30 shade and 30 sun leaves were collected from each (10) forest tree species. In the period of May to September 2014, per species (12, including two conifers) 15 shade and 15 sun leaves were taken at each sampling occasion. In the case of coniferous trees, the author analyzed 3 branches of leaf, estimating their volume to 300 pieces. The collected sun and shade leaves were homogenized and considered as a representative sample. During the examination of the diurnal change (July 7, 2014), she sampled sun-, shade- and semi-shade leaves separately for the Turkey oak. By standardizing the sampling conditions, the effects of external parameters influencing the antioxidant properties of the leaves and the deviations due to the inhomogeneity of the foliage were reduced.

The *bark samples* originated from the forest of Soproni Tanulmányi Erdőgazdaság Ltd. For each species, sampling was performed from a representative log, except for the beech, where a total of 3 logs were sampled. In the case of hornbeam, the whole bark, in the case of beech, the inner bark and at other tree species, inner and outer barks were also sampled.

At leaf samples the use of microwave pretreatment for the inactivation of polyphenol-oxidizing enzymes was an important part of the preparation of the sample. In case of bark samples pretreatment was omitted (except for beech).

In the course of optimizing the extraction solvent and technique, three kinds of solvents (methanol: water 80:20 v/v, ethanol water 80:20 v/v, water) and three methods (ultrasonic, microwave, magnetic stirring) were used.

2. Test methods

From the extracts the antioxidant capacities (DPPH-, FRAP- and ABTS-method) and the amount of phenolic compounds (total phenolic, total flavonoid, total flavan-3-ol-content) were determined by spectrophotometric methods. The HPLC-PDA-MS/MS technique was used for the identification and qualitative assessment of polyphenolic antioxidants from selected tissues (beech bark, hornbeam leaf). In the case of beech bark, the relative quantity of the polyphenols has been determined during the HPLC-PDA-MS/MS measurements using MRM transitions.

3. Statistical evaluations

Data was evaluated using Microsoft Office Excel 2010 (Microsoft Corp., Redmond, USA) and STATISTICA 11 (StatSoft, Tulsa, USA).

Results and Discussion

1. Investigation of leaf samples

a) The effect of microwave pretreatment

During the first leaf tests, parallel sample preparation was applied to find out the effect of the inactivation of polyphenol-oxidizing enzymes on the antioxidant content. Total polyphenol content and antioxidant capacity (DPPH and FRAP) were determined from the leaves.

In the case of beech leaf, the polyphenol profile of the extracts of microwaved and untreated samples was determined by HPLC-PDA-MS/MS to verify whether degradation products could be detected. It has been found that the number of polyphenolic compounds on the chromatograms is higher in the treated samples.

Since at most of the examined tree species, microwave treated leaves had higher polyphenol content and improved antioxidant capacity (or treated leaves weren't worse than untreated leaves for some species, e.g. beech, Oriental hornbeam, Norway maple), the effect of microwave enzyme inactivation is clearly effective for the preservation of leaf antioxidants of the examined forest species.

b) Optimizing of extraction solvent

The aim of the experiment was to find the ideal solvent mixture that allows the most antioxidant compounds to be derived from the leaf samples of deciduous and evergreen trees, taking into account economic aspects and further analyses of the extracts. In the case of 12 forest tree leaf samples, during the optimization of extraction solvent composition, the extracts were made from the leaf samples of May 2014 with three kinds of solvents (methanol: water 80:20 v/v, ethanol: water 80:20 v/v and water). Based on the results and economical considerations, the most remarkable solvent mixture was methanol:water 80:20 v/v, to extract the leaf samples.

c) Optimizing of sampling time

In this course the samples were taken from the same points of Turkey oak's foliage at 5 different times in one day in July 7, 2014, and the diurnal changes of the antioxidant parameters depending on the solar access were investigated. Among the measured parameters, the total flavonoid and total flavan-3-ol-content showed differences

among the leaf types. Of course, these results relate only to Turkey oak, while other tree species may have other effects. On the basis of the results, in the further studies, sun and shade leaves were collected in one time of sampling and resulted in a homogeneous "average" sample. The sampling was done henceforth between 8 and 11 a.m. The leaf sampling has been standardized, and any systematic errors caused by intra-day cycles have been eliminated.

d) Seasonal changes of antioxidant compounds of leaves

In the course of these measurements, antioxidant capacities and phenolic compounds of the methanolic extract from 12 Hungarian forest tree species were examined in the period from May to September 2014. The results of the ABTS measurement show that the highest values were observed in May and June in most species. In the case of FRAP there was another tendency, the highest values were in August and September, but in both methods the best European hornbeam, sweet chestnut, downy oak and Turkey oak had the best values. For the DPPH, a significant increase in antioxidant capacity can only be established in the case of poplar. For other species, there is no clear trend between the months.

On the whole, there is another tendency for each method during the vegetation period, which can be explained by the different selectivity of the methods. Thus a summarized evaluation of these methods is needed to obtain a comprehensive measure of the overall antioxidant efficiency of the leaf extracts. This was achieved by a scoring system, assigning 0 points to the weakest values and 1 to the best values within each antioxidant capacity method. In the ABTS and FRAP methods, the lowest value was scored 0 and the highest value was cored 1. For the DPPH values, opposite scoring was used because the lowest IC_{50} value (score: 1) represent the highest antioxidant capacity, and the highest IC_{50} (score: 0) represent the weakest antioxidant power. The DPPH, FRAP and ABTS scores for each sample were summed up to obtain a measure of their overall antioxidant efficiency. The species with the overall best antioxidant power were European hornbeam, sweet chestnut, and Turkey oak. For most species, August/September samples have the highest overall antioxidant capacity. Exceptions to this are Scots pine (June), black pine (July) and Turkey oak (May).

In the case of phenolic compounds, the total phenol content grows from spring to fall, while flavonoid and flavan-3-ol values vary depending on the species. The total phenol content was the highest at the same species (European hornbeam, sweet chestnut, Turkey oak, downy oak) like at antioxidant capacities. Therefore in the case of

these species polyphenolic compounds can be responsible for the antioxidant effect.

e) Separation and identification of European hornbeam leaf polyphenols by HPLC-PDA-MS/MS method

Of the selected species, the European hornbeam showed the highest antioxidant capacity values. Therefore, in the August leaf extract, it was determined which compounds could be responsible for the antioxidant effect. Altogether 171 compounds were described, and 92 were determined including phenolic acids, ellagitannins, gallotannins, flavonoids, catechins, procyanidins, and many other unknown compounds. The most abundant compounds in the best DPPH and FRAP antioxidant capacity August extracts were chlorogenic acid, ellagic acid, ellagitannins, myricetin-, luteolin-, quercetin- and apigenin glycosides, which are supposed to be mostly responsible for the excellent antioxidant properties. The large number of polyphenolic compounds and the significant antioxidant capacity that characterizes the leaf extract of European hornbeam, it can be said that the leaves of European hornbeam are a promising renewable source for the extraction of antioxidant compounds.

2. Investigation of bark samples

a) The effect of microwave pre-treatment

The effect of microwave pre-treatment was investigated in the case of inner and outer bark of black locust, white poplar, sessile oak, and in the case of inner bark of beech based on the extracts' totalphenol content and antioxidant capacity (DPPH-, FRAP- and ABTS-method). From the experiments, it can be determined whether there is no significant difference between the totalphenol content and the antioxidant capacity of the treated and untreated samples due to the microwave pre-treatment or the decrease of these values could be observed. Among the species, the inner bark of beech is an exception, where in each case the pretreatment had a little positive effect on polyphenol content and antioxidant capacity. The pretreatment has also a positive influence on ABTS values at oak's outer bark. From this series of measurements, it can be concluded that this pretreatment is strongly dependent on species in the case of bark samples. For some species, it does not cause significant changes in the measured parameters or it reduces the amount of antioxidant substances in other species. In the latter case, it is probably not possible and not necessary to inactivate polyphenols oxidizing enzymes by this method, and even because of the excessive microwave action, the compounds responsible for antioxidant

properties are also damaged and thus no longer contribute to the antioxidant effect of the tissue. Based on these knowledge, the microwave treatment (except beech) was not applied for the bark samples.

b) Optimization of extraction method and solvent

The optimization of extraction solvent and method were done in the case of beech bark. During the extracts' preparation different extraction methods (sonication, stirring and microwave assisted extraction) and three solvent systems (methanol:water 80:20 v/v, ethanol:water 80:20 v/v, and water) using different time/temperature schedules were compared. The most optimal method and solvent were selected based on the antioxidant capacity values. On the basis of the results of totalphenol measurements, the best extraction solvent was the ethanol containing mixture at room temperature. Among the methods, the microwave assisted extraction was the most effective, followed by ultrasonic and stirring method. Based on the values of the antioxidant capacities, the ethanolic extracts at room temperature were richer in antioxidant compounds than the methanolic extracts made under same conditions. However, with increased pressure and temperature parameters, water can also become an effective solvent such as alcoholic mixtures. Based on this statement, it will be possible to implement environmentally friendly extraction technology in the future. It is also important to note, that one method of characterizing antioxidant parameters is not sufficient for a plant extract. Compounds responsible for DPPH antioxidant capacity can be obtained by ultrasonic method, while in the case of FRAP, the microwave assisted extraction, and in the case of ABTS , microwave and stirring proved to be the most effective extraction procedure. This also supports the principle, that one method is not suitable for modelling processes in the living organism. Therefore, it is very important to apply more methods to obtain a more accurate image of the antioxidant properties of the plant extracts.

c) The HPLC-MS/MS separation and identification of beech bark polyphenols

The separation and identification of beech bark polyphenols was performed from the ethanolic extract of microwave extraction (on 120°C for 20 min). We can find some information on beech bark polyphenols, but such a detailed and high resolution experiment has not yet been carried out. Altogether 37 compounds, including (+)-catechin, (-)-epicatechin, quercetin-*O*-hexoside, taxifolin-*O*-

hexosides (3), taxifolin-*O*-pentosides (4), B-type (6) and C-type (6) procyanidins have been tentatively identified. The extracts showing the highest totalphenol levels, were further analysed, the quantitative determination of the identified compounds was also performed using MRM transitions. The highest amounts of (+)-catechin and (-)-epicatechin were obtained for microwave assisted methods at 120 °C. For the extraction of procyanidins, sonication proved to be the best method using the ethanol:water 80:20 v/v mixture. After 20 min a decrease of procyanidin content was measured when using solvent mixtures containing alcohol. As in the case of catechins, it can be observed that the procyanidins decompose in the aqueous extracts by increasing the duration, and this also contributes to the reduction of totalphenol content. In the case of taxifolin glycosides microwave assisted extraction proved to be the best method. Overall the choice of extraction method, circumstances and solvent system favors the extraction of different types of compounds from beech bark. Water is proved to be surprisingly efficient with the use of microwave assisted extraction. The use of overpressured high temperature water for the extraction of beech bark could open new possibilities for the implementation of green extraction procedures.

d) Characterization of antioxidant parameters of different barks

Characterization of antioxidant parameters of inner and outer bark samples was performed on 7 species. Antioxidant capacities (DPPH, FRAP and ABTS method) and the amount of phenolic compounds have been determined. Based on the results, the best antioxidant capacities were measured in the case of sweet chestnut (outer bark, DPPH: 2.80 μg/ml), European larch (outer bark, DPPH: 5.84 μg/ml) and black cherry (outer bark, DPPH: 12.0 μg/ml). The lowest value was determined in the case of black poplar (outer bark, DPPH: 30.2 μg/ml). In the case of outer bark, the highest totalphenol levels were measured in the case of European larch, sweet chestnut and black cherry with 121; 89,0; 70.0 mg Q/g d.w. values respectively, while in the case of inner bark, the black cherry was followed by European larch and birch with 139, 107 and 76.6 mg Q/g d.w. respectively. The most flavonoid contents were also determined in the case of outer bark of sweet chestnut and at the inner bark black cherry, while the most flavan-3-ol-content were typical at European larch (outer bark) and at black cherry (inner bark). The bark of these species can be a promising raw material in the future for practical uses.

In the case of bark samples I determined the overall antioxidant capacity of the all outer and inner bark samples (11), by the similar

evaluation used for leaves. Based on the scoring system, among the inner bark extracts, the species with the highest antioxidant capacity was the sweet chestnut, European larch, black cherry and among outer bark extracts sweet chestnut and European larch. The bark of the listed species could be a promising raw material for future applications.

Theses

1. I have performed first a comparative study of the leaves of 12 species of forest tree species in Hungary (European beech, European hornbeam, downy oak, poplar, black locust, Norway maple, Turkey oak, pedunculate oak, sessile oak, Scots pine and black pine). During the comparative study, the antioxidant capacity values (DPPH, ABTS, FRAP) and the quantity of major polyphenol groups (total polyphenols, total flavonoids, total flavan-3-ols) were measured. I have found that there is a need to use several methods for the complex assessment of the overall antioxidant properties of leaf samples in the case of investigated forest tree species. Using the data of the seasonal changes of DPPH, FRAP and ABTS I generated a scoring system, assigning 0 points to the weakest values and 1 to the best values within each antioxidant capacity method, using linear approximation between lowest and highest values. In the ABTS and FRAP methods, the lowest value was scored 0 and the highest value was scored 1. For the DPPH values, the opposite scoring system was used. Finally the DPPH, FRAP and ABTS scores for each sample were summed up to obtain a measure of their overall antioxidant efficiency. I found that the species with the overall best antioxidant power were European hornbeam, sweet chestnut, Turkey oak and downy oak. For most of the examined species, the August/September samples had the highest overall antioxidant capacity, except for Scots pine and Turkey oak.
2. I have described and characterized first the polyphenolic compounds in the European hornbeam leaf by high performance liquid chromatography and coupled tandem mass spectrometry. Altogether 171 compounds were described, and 92 were determined including phenolic acids and derivatives, gallotannins, ellagitannins, flavonoid glycoside, eucaglobulin and catechins. The rest of the compounds was characterized by MS/MS spectra only. The list of the compounds and the significant antioxidant capacity that characterizes the leaf extract

of European hornbeam, it can be said that the leaves of European hornbeam are a promising renewable source for the extraction of antioxidant compounds, which may be even pharmaceutical raw materials in the future.

3. I have performed first the comparative study of the antioxidant capacities (FRAP, ABTS, DPPH) and the main polyphenol groups (total polyphenol, total flavonoid, total flavan-3-ol) of 11 bark of forest tree species (European hornbeam, black cherry, sweet chestnut, black poplar, common birch, European larch, Scots pine, European beech, black locust, sessile oak, white poplar). I have concluded that in the case of bark of the investigated forest species, several different methods need to be used to estimate the antioxidant properties. This was achieved by a scoring system, applied to inner and outer bark samples separately. Score 0 was assigned to the weakest values and 1 to the best values within each antioxidant capacity method. In the ABTS and FRAP methods, the lowest value was scored 0 and the highest value was scored 1. For the DPPH values, the opposite scoring system was used. The DPPH, FRAP and ABTS scores for each sample were summed up to obtain a measure of their overall antioxidant efficiency. Based on the scoring system, among the inner bark extracts, the species with the highest antioxidant capacity was the sweet chestnut, European larch, black cherry and among outer bark extracts sweet chestnut and European larch.

4. I have described and characterized as first the polyphenolic compounds in the inner bark of beech by high performance liquid chromatography/ tandem mass spectrometry. Altogether 37 compounds were separated and the following have been tentatively identified: (+)-catechin, (–)-epicatechin, procyanidin B dimer isomers (6), procyanidin C trimer isomers (6), taxifolin-*O*-hexoside isomers (3), taxifolin-*O*-pentoside isomers (4), 3 catechin derivatives, quercetin-*O*-hexoside, coniferyl-alcohol-*O*-hexoside-*O*-pentoside, 2 syringic acid-di-*O*-hexoside, coumaric acid-*O*-dihexoside and 2 coniferin isomers. The rest of he compunds were characterized only by MS/MS spectra.

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