

Phenolic Compounds of *Pinus brutia* Ten.: Chemical Investigation and Quantitative Analysis Using an Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry with Electrospray Ionization Source

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(Received February 28, 2013; Revised June 10, 2013; Accepted July, 12 2013)

Abstract: In this study, phenolic content of *Pinus brutia*'s bark was examined using an ultra-performance liquid chromatography tandem mass spectrometry with electrospray ionization source (UPLC-ESI-MS/MS) working in multiple reaction monitoring mode. Ultrasonic extraction method with 50% ethanol solution was used for the extraction of bark. The bark of *Pinus brutia* consisted of 15 compounds: gallic acid, gentisic acid, protocatechuic acid, 4-hydroxy benzoic acid, catechin hydrate, vanillic acid, caffeic acid, vanillin, *p*-coumaric acid, ferulic acid, myricetin, resveratrol, luteolin, naringenin, kaempferol. Major compound detected was catechin hydrate (28.305 mg 100 g⁻¹ extract). The phenolic compounds of *Pinus brutia* extract and pycnogenol were compared, and it is shown that both of them consisted of considerable amount of phenolic compounds.

Keywords: *Pinus brutia*; Pycnogenol; Phenolics; UPLC-ESI-MS/MS

1. Introduction

Pinus (Pinaceae), with over 100 widely known species, is the widest extant genus of conifers [1, 2, 3]. Pines are economically an important source of wood, paper, resins, charcoal, food (particularly seeds), and ornamentals [3, 4]. Other characteristic properties of the pines are the closeness of scales, shapes and lengths of the flower buds. The inner part of the bark, known as "cambium", is also edible in some countries, such as pine bark bread (pettuleipä) made with rye flour in Finland and "tallstrun tea" prepared with green pine needles in Sweden [5]. The natural distribution of the genus is restrained to the Northern Hemisphere except for one population of *P. merkusii* located just south of the equator in Sumatra [6]. However, species such as *P. caribaea*, *P. patula*, *P. pinaster*, and *P. radiata* are cultivated worldwide [4].

The genus *Pinus* is cultivated in Turkey. There are five species of the genus *Pinus*; *P. brutia* Tenore (Turkish pine), *P. halepensis* Miller (Aleppo pine), *P. nigra* J.F. Arnold (European black pine), *P. pinea* L. (stone pine, umbrella pine), and *P. sylvestris* L. (Scots pine). The Turkish Ministry of Forestry is making use of only three species of this genus *Pinus* (*P. brutia*, *P. nigra*, and *P. Sylvestris*) in order to produce timber [7]. Although *Pinus* species have economic significance in pharmaceutical and cosmetic sectors, the remnants of the trees after timber production are not much in use [7]. Turpentine has been known to have a long history of healing mostly as topical counter irritants for the treatment of rheumatic disorders and muscle pain and pine bark extract is used in anti-aging cosmetics [8].

Pycnogenol is a standardized water extract of French maritime pine (*Pinus maritima*) bark, produced by validated extraction procedure. Although its chemical composition is still not completely

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elucidated, the main constituents of Pycnogenol are known to be phenolic compounds, broadly divided into monomers (catechin, epicatechin and taxifolin) and condensed flavonoids (classified as procyanidins/proanthocyanidins). Pycnogenol also contains phenolic acids. These are present both as free acids - *p*-hydroxybenzoic, protocatechuic, gallic, vanillic, *p*-coumaric, caffeic and ferulic acid - and as glucosides - *p*-hydroxybenzoic and 4 β -D-glucoside and vanillic acid 4- β -D-glucoside – as well as glucose ester - 1-(*p*-coumaroyl) β -D-glucose and 1-(feruloyl) β -D-glucose. Vanillin and free glucose are also found in Pycnogenol in minute quantities [9,10]. Pycnogenol has been reported to possess various beneficial effects for human health [10,11,12] as it significantly improved the legs' heaviness and subcutaneous edema; the venous pressure was also significantly reduced by the Pycnogenol treatment, thus adding further clinical evidence to its therapeutic efficacy in patients with chronic venous insufficiency (CVI). Pycnogenol was effective, it either stabilizes the collagenous subendothelial basal membrane or scavenges the free radicals, or works by a combination of these activities. Therefore it can be recommended both for prevention and treatment of CVI and related veno-capillary disturbances. In the very recent literature survey of pycnogenol, there were investigations about antioxidant activity of pycnogenol in health promotion, bioactive food as dietary interventions for arthritis and related inflammatory diseases [13], antioxidant activities of pycnogenol [7], dietary supplements, immune modulation, and diabetes control [14], dietary supplements and herbs in diabetes and its prevention [15], anti-inflammatory actions of pycnogenol: diabetes and arthritis [16], neuroprotective effect of pycnogenol following traumatic brain injury [17]. Moreover, one of the last published reports was about fruit juice enriched with pine bark extract [18]. All these investigations demonstrated that pycnogenol is a miracle substance for the human being.

Phenolic compounds are natural antioxidants and they are considered to have a preventive role in the development of cancer and heart disease [19]. Phenolic acids are a group of phenolic compounds biosynthesised by the shikimate pathway [20]. This class of phenolic compounds exhibits various physiological activities, including antibacterial, anti-inflammatory and anticarcinogenic [21,22]. Researches about biological and pharmacological activities have also been documented for phenolic compounds, including free radicals scavenging, apoptosis of cancer cells [23,24], antiherpetic, antihuman immunodeficiency virus (HIV) reverse transcriptase and anti-HIV activity [25,26].

High performance liquid chromatography/tandem mass spectrometry (LC-MS/MS) has been successfully used for characterizing and identifying polyphenol compounds in complex samples [27].

In this study authors aimed to reveal and to compare phenolic compounds of *Pinus brutia* Ten. and pycnogenol.

2. Materials and Methods

2.1. Plant Source

The bark samples of *P. brutia* Ten. were collected from Mugla, Turkey province, between May and June in 2012. The specimens are dried and kept in Department of Biology, Mugla Sıtkı Koçman University, Mugla (Turkey). The dried samples of the plant materials were stored at 4-8 °C in refrigerator until the extraction process.

2.2. Chemicals

Standards (homogentisic acid, 3,4-dihydroxybenzoic acid (protocatechuic acid), gentisic acid, *p*-hydroxy benzoic acid, catechin hydrate, vanillic acid, caffeic acid, vanillin, catechin gallate, *p*-coumaric acid, ferulic acid, *trans*-2-hydroxy cinnamic acid, myricetin, resveratrol, *trans*-cinnamic acid, luteolin, naringenin, kaempferol, hesperetin, chrysin, chlorogenic acid, gallic acid, pyrogallol) were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). They were of at least 98% purity. Methanol was of LC–MS grade and formic acid was 98-100% purity purchased from Merck (Darmstadt, Germany). All other chemicals were of analytical reagent grade and purchased from Merck (Darmstadt, Germany). HPLC grade water (18.2 m Ω) was purified using a Milipore Milli-Q (Bedford, MA, USA) system that involves reverse osmosis, ion exchange and filtration steps.

2.3. Instrumentation and experimental conditions

A Waters Acquity ultra-performance liquid chromatography (UPLC) equipped with a Waters (Milford, USA) BEH C18 column (100 mm×2.1 mm, 1.7 μ m particle size) and a Waters Xevo TQ-S Triple Quadrupole tandem mass spectrometer with an electrospray ionization source were used (Waters Corporation, Milford, MA USA). Data acquisition was performed with Waters Xevo TQ-S quantitative analysis TargetLynx software and data processing was executed using MassLynx mass spectrometry software. Linear gradient elution with a mobile phase comprising water acidified with 0.05% formic acid and 0.05% ammonium formate (solvent A) and acetonitrile acidified with 0.05% formic acid (solvent B) commenced at 99:1 (A:B) and changed to 70:30 (A:B) in 10 min, then from 70:30 (A:B) to 5:95 (A:B) in 2 min and then changed from 5:95 (A:B) to 99:1 (A:B) in 2 min. and finally goes 99:1 (A:B) for 6 min. Run time is 20 min. Flow (0.650 mL min⁻¹) from the liquid chromatography was injected directly into the ESI source, maintained at a temperature of 500 °C and mass detector was measured under the optimized parameters indicated in (2.3). Mass spectra were acquired in negative ESI mode, and elaborated using the Masslynx software and multiple reaction monitoring (MRM) mode.

The multiple reaction monitoring (MRM) mode was applied to monitor the transitions of quantifier ion to qualifier ions (the parent-to-daughter ions transitions) of m/z 125.01 \rightarrow m/z 69.10, 79.04, 81.02 for pyrogallol, m/z 167.03 \rightarrow m/z 123.03, 122.08, 108.00 for homogentisic acid, m/z 153.06 \rightarrow m/z 108.00, 81.01, 91.01 for 3,4-dihydroxybenzoic acid (protocatechuic acid), m/z 153.05 \rightarrow m/z 109.04, 108.03, 81.00 for gentisic acid, m/z 136.98 \rightarrow m/z 93.03, 65.10 for *p*-hydroxy benzoic acid, m/z 137.00 \rightarrow m/z 91.93, 107.94, 136.00 for 3,4-dihydroxybenzaldehyde, m/z 288.88 \rightarrow m/z 109.15, 124.99, 245.26 for catechin hydrate, m/z 166.98 \rightarrow m/z 151.97, 108.03, 123.03 for vanillic acid, m/z 179.10 \rightarrow m/z 135.14, 107.10, 133.9 for caffeic acid, m/z 150.99 \rightarrow m/z 136.00, 92.02, 108.10 for vanillin, m/z 163.01 \rightarrow m/z 119.04, 93.00, 117.01 for *p*-coumaric acid, m/z 193.03 \rightarrow m/z 134.06, 178.00, 149.02 for ferulic acid, m/z 441.00 \rightarrow m/z 168.98, 288.97 for catechin gallate, m/z 163.04 \rightarrow m/z 119.04, 117.01, 93.07 for *trans*-2-hydroxy cinnamic acid, m/z 316.90 \rightarrow m/z 107.07, 137.01, 150.97 for myricetin, m/z 227.01 \rightarrow m/z 143.01, 159.05, 185.03 for resveratrol, m/z 146.98 \rightarrow m/z 103.03, 62.18 for *trans*-cinnamic acid, m/z 284.91 \rightarrow m/z 107.01, 133.05, 151.02 for luteolin, m/z 270.98 \rightarrow m/z 107.00, 119.04, 150.97 for naringenin, m/z 284.90 \rightarrow m/z 158.97, 117.10, 227.14 for kaempferol, m/z 301.02 \rightarrow m/z 108.01, 136.00, 163.99 for hesperetin, m/z 252.99 \rightarrow m/z 63.05, 107.05, 142.99 for chrysin, m/z 353.02 \rightarrow m/z 191.01, 179.09, 161.02 at negative ionization mode. Confirmation of compounds was achieved through two or more daughter ions.

The optimal instrument parameters of the mass spectrometer (MS) were as follows: Desolvation gas temperature, 500 °C; gas flow, 16.7 L min⁻¹; nebulizer pressure, 7 bar; capillary voltage, 2000 V; cone voltage 25 V. Mass spectra were acquired in negative electrospray ionization (ESI) mode and elaborated using the MassLynx mass spectrometry software. The instrument was used in the tandem MS mode.

2.4. Preparation of the stock solutions

The stock solutions (10 g 100 mL⁻¹) were dissolved in methanol. All the stock solutions were kept at -18 °C.

2.5. Preparation of standard samples for calibration curves and quality control (QC)

The calibration curves were prepared at the concentration levels of 0.050, 0.100, 0.250, 0.500, 1.000, 2.500 mg L⁻¹. The standard solutions were stored at 4 °C. QC samples were prepared at 0.250 mg L⁻¹.

2.6. Sample preparation

The dried samples were grounded to fine powder in a waring stainless steel blender and weighed accurately in a digital balance (Mettler Toledo XP205, Greifensee, Switzerland). Then, fine powder pine

bark (100 g) was successfully extracted with 50% ethanol solution (300 mL) at room temperature three times for one day with ultrasonic waterbath. After combining extracts it was filtered, the organic phases were evaporated under vacuum by a rotary evaporator (Heidolph, Germany). Later aquatic extract was frozen at -18°C and then it was lyophilized (Christ Freeze Dryer Alpha 1-4 LD plus). Ten grams of dry residue was dissolved in methanol (100 mL). Then the extract were filtered with $0.20\ \mu\text{m}$ PTFE and injected to UPLC-ESI-MS/MS.

2.7. Method Validation

The analytical method was validated according to the European Medicines Agency (EMA) guidelines relating to the validation of analytical methods [28].

The method based on the characteristic fragmentation reactions of phenolic compounds was highly specific with no any other peak interfering at the marker compounds in the MRM chromatograms. The intra-day accuracy and precision were calculated by analysing three samples of compounds at midlevel of concentration, namely, $0.250\ \text{mg mL}^{-1}$, on the same day. Inter-day estimates were performed over three consecutive days. The standard deviation was $<5\%$. The calibration graphs were obtained by plotting the area obtained from external standard against the known concentration of external standard (for each compound) (S1-17). The limit of quantification (LOQ), defined as the lowest concentration of compound quantifiable with acceptable accuracy and precision, was determined by injection of a series of diluted standard solutions until a signal-to-noise ratio of 10 was attained. Validation data of the method developed for quantitative analysis of compounds were displayed in supporting information S18.

3. Results and Discussion

In the chemical investigation of the ethanol extract of the bark of Turkish *Pinus brutia*, fifteen phenolic compounds were detected. Some details of the analysis were given in Table 1 and supporting information S18.

All the compounds were characterized using ultra-performance liquid chromatography tandem mass spectrometry with electrospray ionization source. Total Ion Chromatogram (TIC) of bark of Turkish *Pinus brutia* was given in Figure 1. Peaks displayed in TIC are numbered to represent the phenolic compounds detected in the extract of bark of Turkish *Pinus brutia*.

In this study catechin hydrate depicted as (5) was evaluated as the major constituent ($28.305\ \text{mg } 100\ \text{g}^{-1}$) then gentisic acid, protocatechuic acid, gallic acid and caffeic acid depicted as (2), (3), (1) and (7) respectively, were determined as ($2.220\ \text{mg } 100\ \text{g}^{-1}$), ($2.551\ \text{mg } 100\ \text{g}^{-1}$), ($0.177\ \text{mg } 100\ \text{g}^{-1}$) and ($0.349\ \text{mg } 100\ \text{g}^{-1}$) respectively in the extract of bark of Turkish *Pinus brutia*.

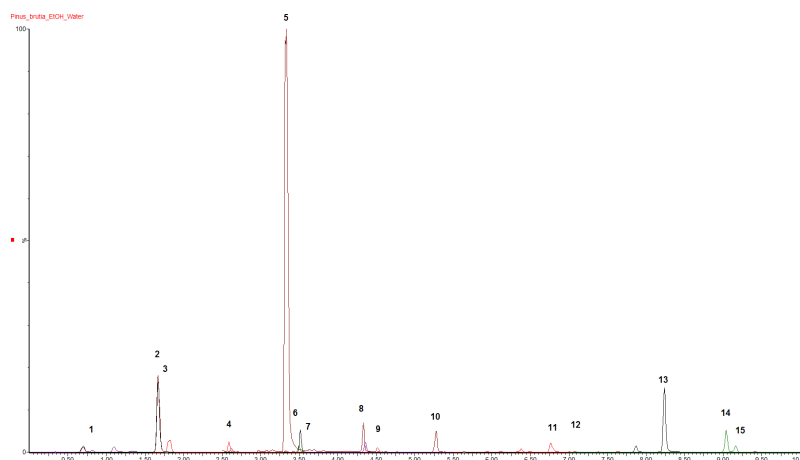


Figure 1. TIC of bark of Turkish *Pinus brutia* : Gallic acid (1), Gentisic acid (2), Protocatechuic acid (3), 4-hydroxy benzoic acid (4), Catechin hydrate (5), Vanillic acid (6), Caffeic acid (7), Vanillin (8), *p*-Coumaric acid (9), Ferulic acid (10), Myricetin (11), Resveratrol (12), Luteolin (13), Naringenin (14), Kaempferol (15).

In the Pynogenol analysis, seventeen phenolic compounds were identified. Evaluated amounts were displayed in the Table 1. All the compounds were characterized using UPLC-ESI-MS/MS. TIC of Pynogenol was given in Figure 2. Peaks displayed in TIC are numbered to represent the phenolic compounds detected in Pynogenol.

Catechin hydrate depicted as (5) was evaluated as the major constituent (25.440 mg 100 g⁻¹) then gentisic acid, protocatechuic acid, gallic acid and caffeic acid depicted as (2), (3), (1) and (7) respectively, were determined as (0.897 mg 100 g⁻¹), (1.884 mg 100 g⁻¹), (0.593 mg 100 g⁻¹) and (0.520 mg 100 g⁻¹) respectively.

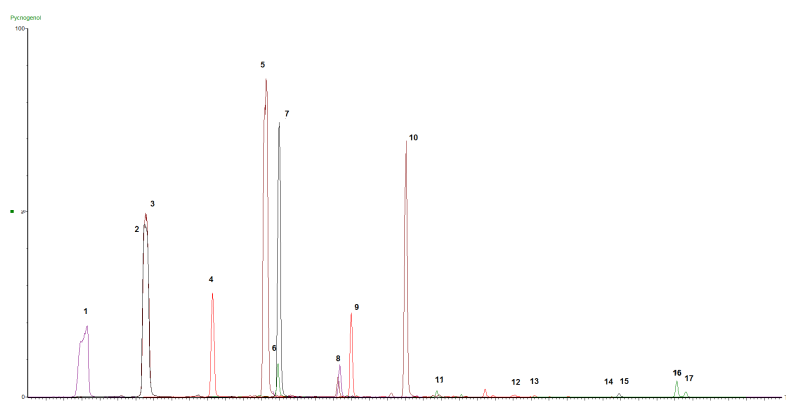


Figure 2. TIC of Pynogenol : Gallic acid (1), Gentisic acid (2), Protocatechuic acid (3), 4-hydroxy benzoic acid (4), Catechin hydrate (5), Vanillic acid (6), Caffeic acid (7), Vanillin (8), *p*-Coumaric acid (9), Ferulic acid (10), Catechin gallate (11), Myricetin (12), Resveratrol (13), *trans*-Cinnamic acid (14), Luteolin (15), Naringenin (16), Kaempferol (17).

Table 1. Conditions applied during UPLC-ESI-MS/MS analysis, and quantified amounts of phenolic compounds for *P. brutia* Ten. and for Pynogenol. Results are given as: mg 100 g⁻¹ extract (\pm SD).

Name	<i>Pinus brutia</i> (mg 100 g ⁻¹ extract)	Pynogenol (mg 100 g ⁻¹ extract)	RT (min.)	Cone (V)	Collision Energy (V)
4-hydroxy benzoic acid	0.332 (\pm 0.009)	0.383 (\pm 0.017)	2.57	10	25, 14
<i>trans</i> -Cinnamic acid	ND	0.019 (\pm 0.003)	8.13	30	10, 10
Resveratrol	0.026 (\pm 0.002)	0.028 (\pm 0.004)	7.05	30	25, 18
Catechin gallate	ND	0.070 (\pm 0.006)	5.82	30	20, 20
Homogentisic acid	ND	ND	1.29	10	20, 20, 10
Gentisic acid	2.220 (\pm 0.051)	0.897 (\pm 0.018)	1.67	10	20, 20, 12
Vanillin	0.169 (\pm 0.019)	0.057 (\pm 0.005)	4.36	30	20, 20, 14
Vanillic acid	0.146 (\pm 0.028)	0.080 (\pm 0.007)	3.47	20	18, 12, 14
Catechin hydrate	28.305 (\pm 0.086)	25.440 (\pm 0.042)	3.32	30	25, 20, 15
Chlorogenic acid	ND	ND	3.44	10	20, 20, 20
<i>p</i> -Coumaric acid	0.057 (\pm 0.011)	0.112 (\pm 0.022)	4.52	5	27, 27, 15
Ferulic acid	0.327 (\pm 0.032)	0.325 (\pm 0.030)	5.28	20	16, 12, 13
Hesperetin	ND	ND	9.66	20	36, 30, 24
Chrysin	ND	ND	11.06	20	25, 30, 25
Protocatechuic acid	2.551 (\pm 0.037)	1.884 (\pm 0.033)	1.66	10	20, 25, 20
<i>trans</i> -2-hydroxy cinnamic acid	ND	ND	6.2	10	25, 25, 22
Gallic acid	0.177 (\pm 0.021)	0.593 (\pm 0.048)	0.82	10	20, 20, 20
Myricetin	0.227 (\pm 0.016)	0.065 (\pm 0.007)	6.75	30	30, 25, 25
Naringenin	0.135 (\pm 0.012)	0.017 (\pm 0.003)	9.04	20	25, 25, 20
Caffeic acid	0.349 (\pm 0.018)	0.520 (\pm 0.027)	3.52	32	23, 23, 24
Pyrogallol	ND	ND	0.92	20	17, 17, 14
Luteolin	0.146 (\pm 0.009)	0.013 (\pm 0.002)	8.22	20	30, 33, 30
Kaempferol	0.036 (\pm 0.003)	0.011 (\pm 0.002)	9.21	10	34, 40, 30

ND: Not detected

The results clearly indicate that bark of Turkish *Pinus brutia* and pycnogenol have similar constitutions and amounts with respect to phenolic ingredients. It was already reported [10,11,12] that pycnogenol holds various beneficial effects for human health. It has high antioxidant activity [7] as a food supplement.

In conclusion, extracts of Turkish *Pinus brutia* Ten. contain considerable amount of phenolic compounds as compared to commercially available pycnogenol. While pycnogenol is considered as a miracle substance for the human being due to phenolic content and antioxidant activity, extract of bark of Turkish *Pinus brutia* can also be used as an alternative to pycnogenol due to its similarity in phenolic contents with pycnogenol.

According to our results, Turkish *Pinus brutia* may be considered as a promising natural phenolic compounds source.

Acknowledgments

The authors are grateful to Food Analysis Application and Research Center in Muğla Sıtkı Koçman University for the UPLC-ESI-MS/MS analysis.

Supporting Information

Supporting Information accompanies this paper on <http://www.acgpubs.org/RNP>

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