Determination of Phenolic Compounds in *Pinus eldarica* by HPLC

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Abstract

Background: **The antioxidant components have been identified in some pine species. Antioxidant properties of proantocyanidins reduce free radicals induced by DNA fragmentation and lipid proxidation and also proanthocyanidines could curb lipid peroxidation.**

Objective: **In this study, we analyzed different parts of *Pinus eldarica* (bark, seed and needle) and assessed their antioxidant contents.**

Methods: **Pine specimens were collected from four different geographic locations in Tehran. The HPLC method (UV detector, C_{18} reverse phase column, 4.6 mm (25 cm, and water/ H_3PO_4/ methanol/ acetonitril as eluant) were employed for evaluating total polyphenols. The wavelength for detection of polyphenolic compounds was 280 nm in this study.**

Results: **The highest range of total polyphenols was detected in the bark of this pine, specially reported a considerable amount of tyrosol in Pinus eldarica. Tyrosol stimulated resistance to oxidative stress and also has anti aging effect.**

Conclusion: **The high amount of total phenolic compounds in *P. eldarica* bark might be attractive for future research considering its health benefits.**

Keyword: **Pinus, Phenolic compound, HPLC, Catechin, Poly phenols**

Introduction

Antioxidant properties of proantocyanidines have been determined in several animal studies. It has been reported that proanthocyanidine supplementation in rats increases their total plasma antioxidant activity [1]. Moreover, proanthocyanidine extracts such as those seen in grape seed, reduce free radicals induced by DNA fragmentation and lipid peroxidation to a substantially greater amount compared to other antioxidants [2]. Human studies have also demonstrated the protective effect of proanthocyanidines against DNA damage in lymphoblastoid cells [3]. In addition, proanthocyanidines could curb lipid peroxidation [4]. Likewise, antioxidant components delay chronic conditions such as atherosclerosis, diabetes and osteoarthritis [5-8]. Appreciable amounts of proanthocyanidines have been reported to be present in different pine species [9 - 12].

This diversity in polyphenol content may account for different health benefits of pine extracts. A well known species of pine (*Pinus pinaster*) contains 65-75% proanthocyanidines in its dietary extract (pycnogenol) [13], and has been used since the time of Hippocrates for therapy of inflammatory diseases [14]. Several studies have been conducted on the evaluation of polyphenolic and proanthocyanidine components in various pine species using liquid chromatography in different geographical areas. A study in Turkey reported the catechin content in the bark of *P. brutia* and other Turkish pines [9]. Two other reports focused on phenolic components of *P. halepensis* and *P. laricio* needles. While the former study measured p- comaric acid, vanilic acid and gallic acid, the latter reported p- coumaric acid, vanillic acid and ferullic acid [10, 11]. Total phenolic compounds have also been studied in Norwegian pine needles [12].

Pinus Eldarica is an evergreen tree which belongs to the *pinacea* family. It has been observed in the middle east and the land around the Caspian sea since 2500 years ago. It typically grows very fast and tolerates heat, wind and dry weather hence it is also named as desert pine. Data on the content of phenolic compounds in *P. eldarica* which is native to Iranian region are lacking. Also, there is no study on the antioxidant components of the Iranian pine bark extract. Therefore, we aimed to determine the phenolic contents in different parts of *P. eldarica* located in Tehran (commonly found in Iran and Afghanistan) by high performance liquid chromatography (HPLC).

Materials and Methods

Reagents and standards

The reagents were pure HPLC grade. Methanol, phosphoric acid, chloroform, acetonitril and sodium chloride from Merck (India), and Milli-Q water were used. Ethyl acetate was purchased from Sinopharm Chemical Reagent (China).

Polyphenol standards: (+) - catechin, (-) - epicatechin, Gallic acid, vanillic acid, *para* coumaric acid, ferullic acid, *ortho* coumaric acid and tyrosol were purchased from Sigma-Aldrich (Germany).

Instrument

HPLC analysis

HPLC analysis was undertaken using Waters 1525 (USA). The system included quaternary pump, column heater, UV detector (Waters 2487) at 280 nm, C_{18} reverse-phase column (4.6 mm (25 cm) with breeze software.

Mobile phase consisted of water 0.2%: H_3PO_4: methanol: acetonitril (96:2:2, v: v: v) (Gradient elution) Temperature was 25 (C and flow rate was 1 mL. min^{-1}. The 20 µl loop was used. UV detector was turned on at least 1 h before sample injection. The chromatography column should be conditioned at least 10 min before analysis.

Plant material

Pine specimens (bark, seed & needle) were collected from four different geographic locations in Tehran: Chitgar forest park (West of Tehran), Lavizan forest park (North of Tehran), Sorkhe-Hesar forest park (East of Tehran) and Kahrizak area (South of Tehran). First, the samples were identified by a botanist (Ms. Maryam Ahvazi). Afterwards, sample collection was completed between June and July, 2010. A voucher specimen of the plant (number 689) is deposited in the herbarium of the Institute of Medicinal Plants, ACECR.

Samples were dried at 25°C and stored at 5°C. Then polyphenols in bark, seeds and leaves of the pines were extracted in 10 random samples.

Preparation of pine extracts

This was performed according to the method which was developed by Masquelier [15]. Briefly, (100 g) of pine bark was grounded. The powder was added to 600 mL of boiling water and cooled down to 20°C and filtered. Then sodium chloride was added to 250 mL of extracting solution. The solution was saturated with sodium chloride and filtered. Then ethyl acetate was added to filtrate (1 mL of ethyl acetate with 10 mL filtrate with). Ethyl acetate was dried with anhydrous sodium sulfate using a rotary evaporator, until the volume reached 1.5 mL. Then three volumes of chloroform were added to the extracted solution and stirred mechanically and the proanthocyanidine participate was formed at the mixture of Ethyl acetate and chloroform. Afterwards, the proanthocyanidine precipitate was collected by filtration. The obtained powder was stored at -20°C.

Preparation of the standard and sample solutions

Standard Solution

A standard solution was prepared by dissolving 10 mg of (+) catechin (sigma), epicatechin (sigma), and other phenolic compounds in 10 mL methanol and diluting this solution with methanol HPLC grade. The range of concentration was between 50 to 1000 µg/ml the standard curve was linear ($R^2 \geq 0.998$).

Sample Solution

Then 20 µL of sample solution was injected to the HPLC and the chromatogram was recorded at 280 nm. First, 20 µl of standard solution was injected and the chromatogram was recorded at 280 nm. Next, we compared the area of the peaks in standard and sample chromatograms and calculated the amount of polyphenols in *Pinus eldarica*. Total phenol was computed from measurement of individually detected phenols.

Statistical analysis

Each experiment was performed at least 3 times. Data were expressed as the means ± SD (standard deviation). Comparison of polyphenols between 3 parts of a pine tree (bark, seed and needle) were performed by ANOVA (SPSS, version 19). Values less than 0.05 were considered significant.

Results

In this study, the phenolic components of bark, seed and needle of *P. eldarica* were investigated by HPLC. The wavelength for detection of polyphenolic compounds was 280 nm in this study. It was the best wavelength to differentiate polyphenols with minimal interference from other simultaneous compounds. Chromatograms obtained from analysis of the bark, needle and seed of the Pinus Eldarica extract are shown in Figure 1. The retention time of catechin and epicatechin were approximately 12 and 13 min, respectively. The total run time of determination was 45 min.

Appropriate sample extraction is of paramount importance in determination of phenolic compounds because the method of extraction should keep chemical changes away. Considering this method more than 8 polyphenols were identified in Pinus eldarica. Total phenolic compounds in bark, seed and needle of the pine are shown in Table 1.

Regression curves of Catechine and Epicatechine were shown in Figures 2 and 3, respectively. Linearity was shown for Catechin and Epicatechin in different concentrations between (50- 1000 μg/ml). Total polyphenols Quantified according to the method of Masquilier and polyphenols were analyzed against standard curves.

The amount of total phenolic compounds is different in various sections of pine tree.

The polyphenol content in the bark of pine is much more than that in its seed and needle. The total phenolic constituents of pine bark were six times more than those of pine seed and also one and a half times more than that of pine needle (Figure 4).

Among individual phenolic components, the levels of catechin, epicatechin (Figure 5) and their dimers were higher than other antioxidants in different parts of pine. In fact, catechin comprised the main phenol constituent in different parts of pine. The content of epicatechins in pine bark and needles were higher than that of its seeds. Also the same amount of catechins were found in the bark and needle.

The amount of catechin dimers in the bark and seed were almost equal and the content of dimers of catechin in pine needles were three fold higher than that of its bark and seed. Tyrosol is also one of the main phenolic compounds present in pine specimens which has important antioxidant and cardioprotective properties. The content of tyrosol in seed is 1.6 fold higher than bark and 2.2 times more than the needle. There is no vanillic acid in the seed and the amount of gallic acid and ortho coumaric acid in the bark and needle are more than in seed.

Discussion

To our knowledge, this is the first study to determine the amount of polyphenols in *P. eldarica* species. The results demonstrate that a significant amount of catechins and epicatechins (subgroup of flavanols) is found in the bark, seed and needle of this pine (0.03%, 0.005% and 0.019% respectively, based on dry weight). There are several studies which report the existence of polyphenols in various pines as well as other plant species. Boros et al., reported the existence of catechin, epicatechin, caffeic acid and *p*-coumaric acid in *Thymus* species [16]. Another study by Pfundestein et al., reported the presence of gallic acid in the fruits of Egyptian medicinal plants (*Terminalia bellerica, Terminalia chebula* and *Terminalia horrida*) [17]. While these studies provide a qualitative report of

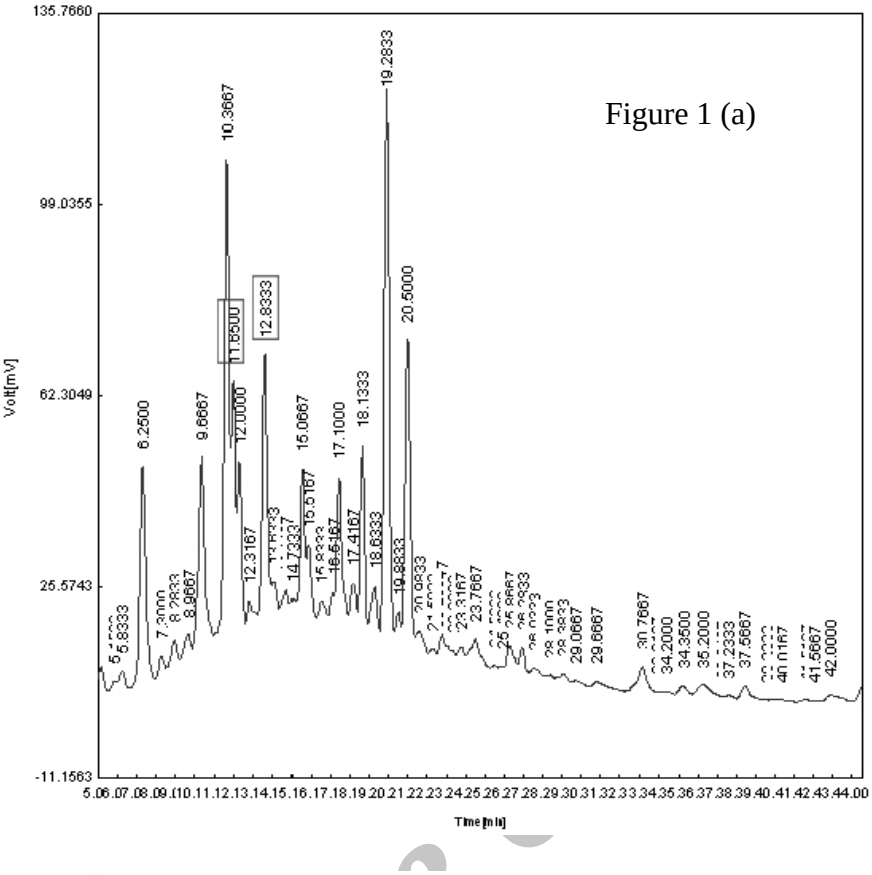


Figure 1 (a)

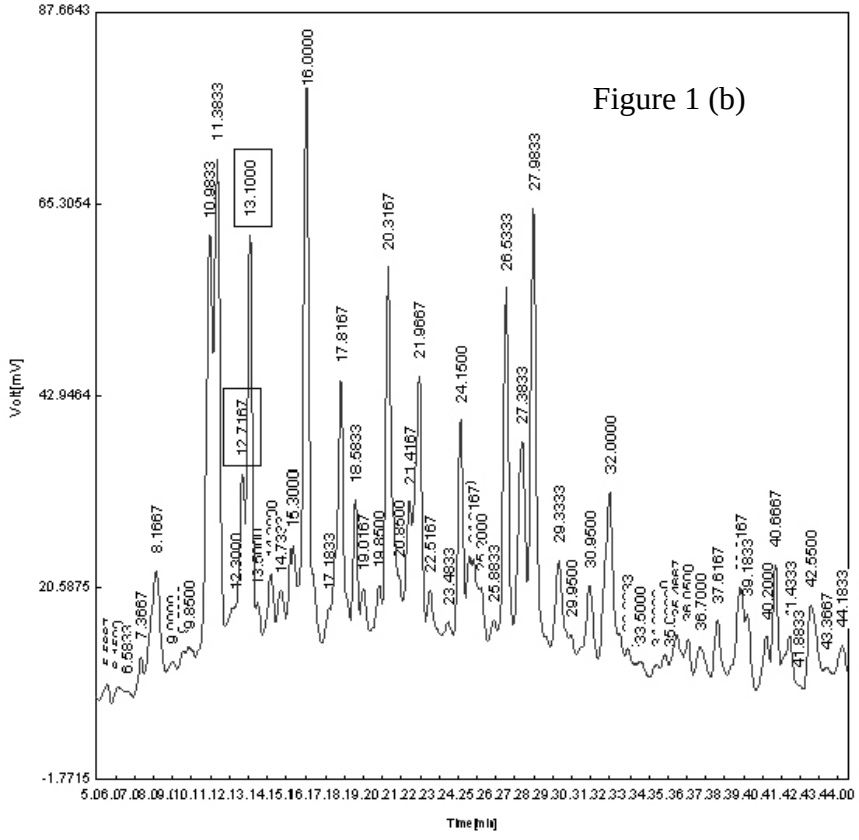


Figure 1 (b)

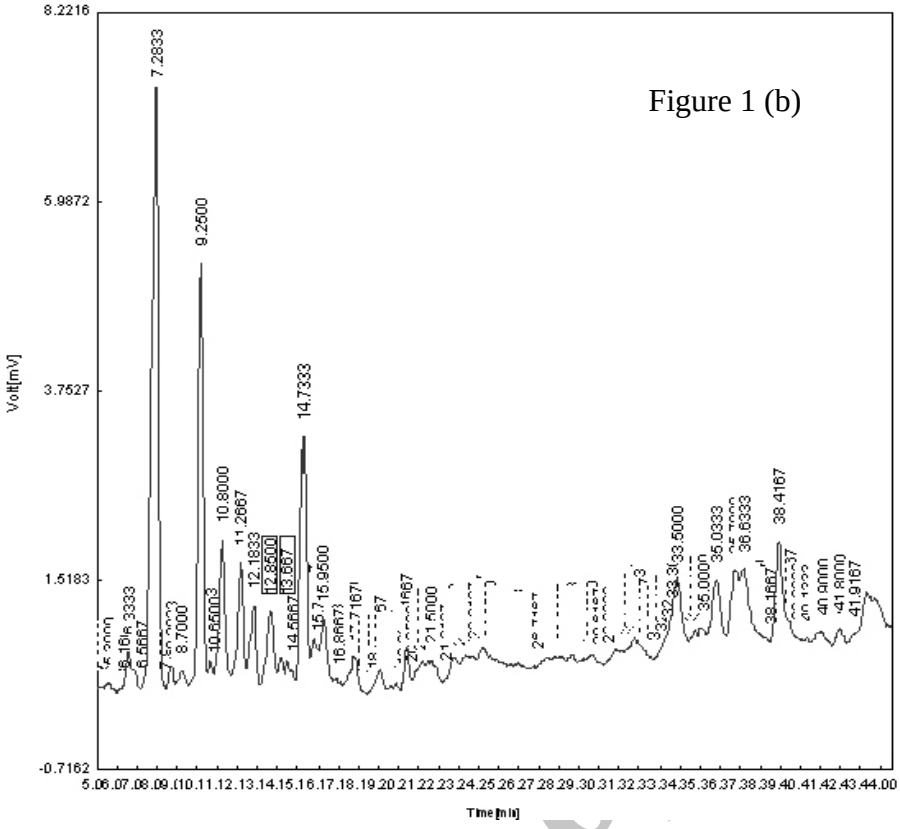


Figure 1- Chromatograms of polyphenolic compounds of (a) bark, (b) needle, (c) seed of Pinus eldarica

Table 1- Different polyphenol contents in Pinus eldarica mean ±SD (%)

Poly phenols (%)	Bark	Seed	Needle
Catechin	9.6 ± 0.13	10.1 ± 0.18	9.6 ± 0.37
Epicatechin	14.4 ± 0.16	10.3 ± 0.18	12.5 ± 0.29
Gallic Acid	6.16 ± 0.03	1.6± 0.09	5.5± 0.08
Vanillic Acid	7.4 ± 0.05	—	4.3 ± 0.11
P. Coumaric Acid	10.4 ± 0.03	1.4 ± 0.12	12.5 ± 0.16
Ferullic Acid	8.1 ± 0.06	1.7 ± 0.21	5.8 ± 0.17)
O. Coumaric Acid	18.2 ± 0.10	0.12 ± 0.02	12.8 ± 0.21
Tyrosol	17.7 ± 0.10	29.1 ± 0.08	13.1 ± 0.10
Dimers of Catechin and Epicatechin	7.87 ± 0.97	7.5 ± 1.06	22.1 ± 0.81
Unknown	0.16 ± 0.1	38.18 ± 0.28	1.8 ± 0.13
Total phenols (ppm)	3024 (70)	483 (27)	2003.9 (40.45)

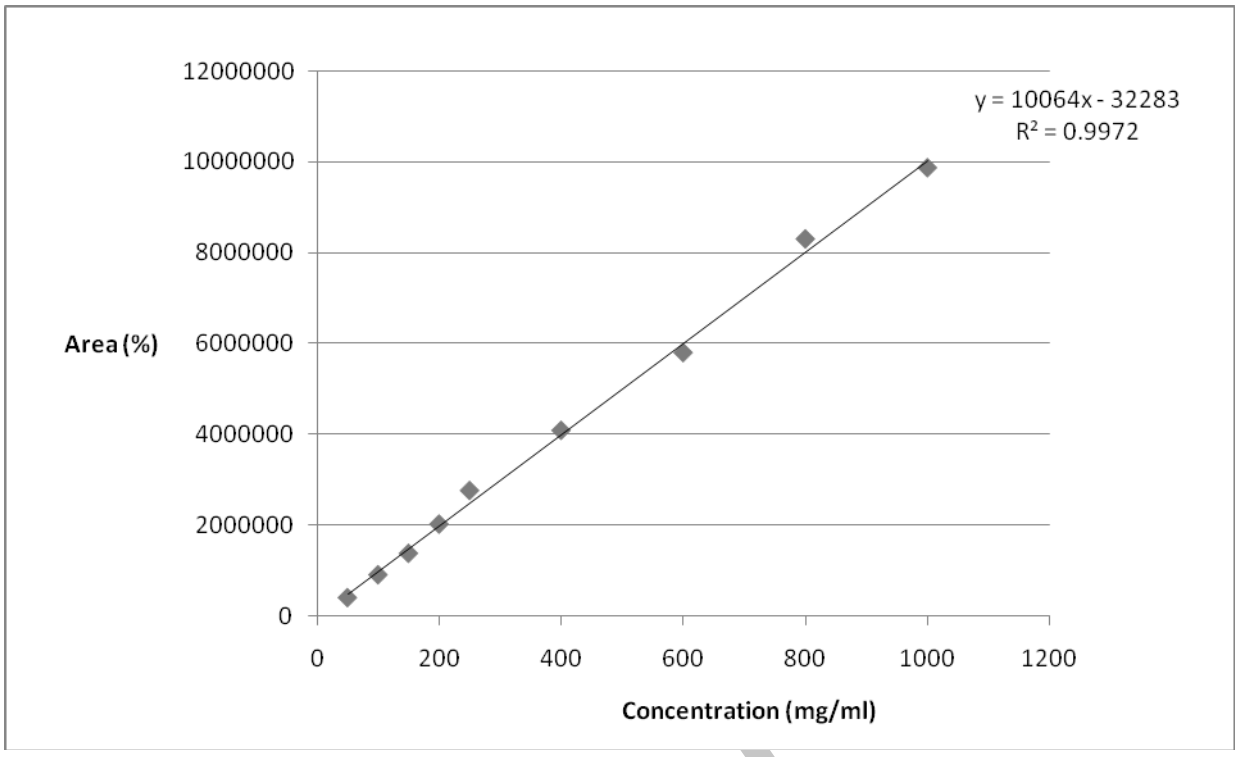


Figure 2- Regression curve of Catechin

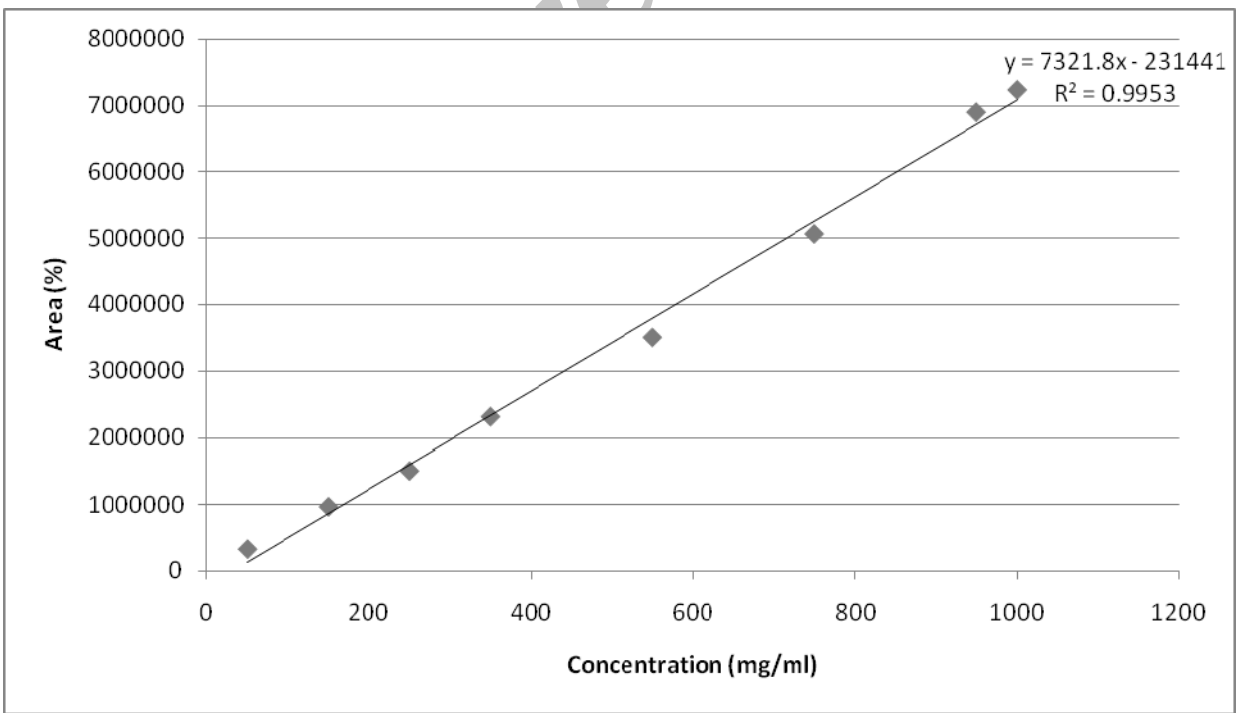


Figure 3- Regression curve of Epicatechin

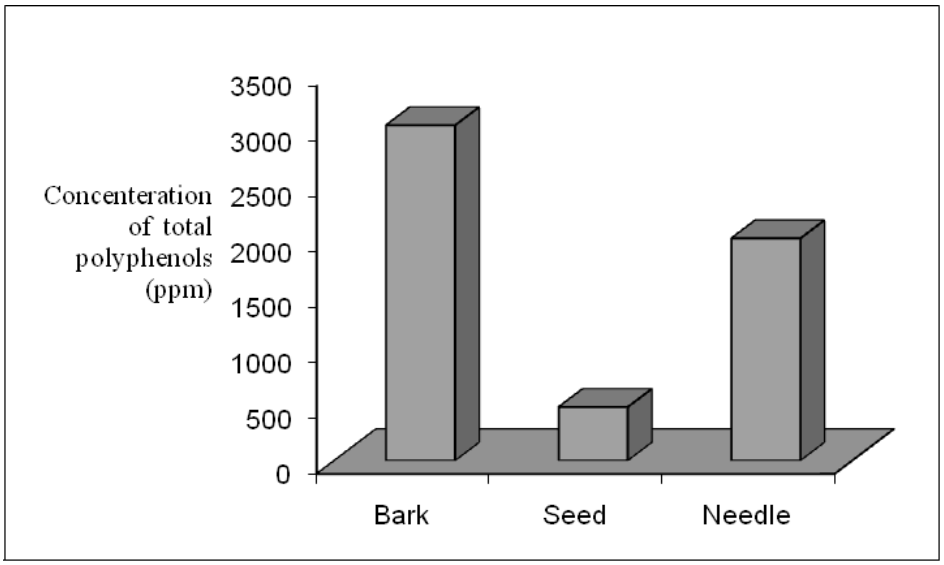
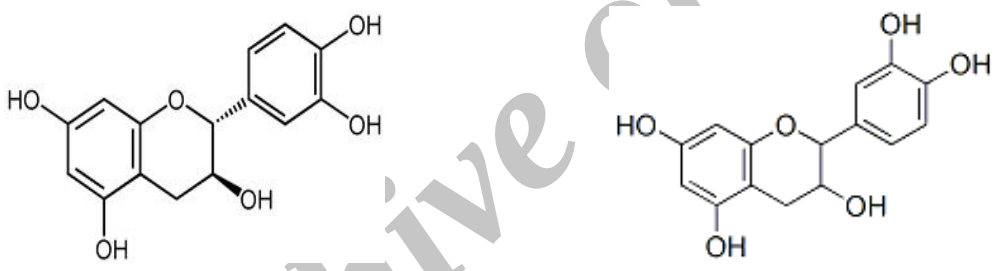


Figure 4- Concentration of total phenolic compounds in bark, seed and needle of *P. eldarica*



(+)-Catechin (-)- Epicatechin

Figure 5 - Structure of (+) -catechin and (-) – epicatechin

polyphenol content in the studied species, there are several other studies which assayed catechin and epicatechin contents quantitatively. For example a recent study from Turkey determined the concentration of two polyphenols (catechin and toxifolin) in the bark of Three different kinds of Turkish pine as well as in commercial pycnogenol [9]. The catechin content of three varieties of pine bark extracts in their study was reported higher than that of ours. (0.13-0.16% vs. 0.03%). However they did not mention other polyphenols in the bark of their studied pines. In addition they did not determine the polyphenol content of other parts in the pines such as their seeds and needles.

We found that the catechin content of Pinus eldarica bark is one and a half times higher than that of *Pinus nigra* (0.03% vs. 0.02%). Another study reported phenolic constituents in cones and berries of the same Turkish coniferous pine species [18]. While, the range

of catechin was 0.3-3.6% in this study, *pinus eldarica* contained 0.005-0.03% catechin.

Notably, there was no tyrosol in the cones and berries of Turkish pine whereas our study reported a considerable amount of tyrosol in Pinus eldarica. This interesting finding may be of importance due to noticeable antioxidant and cardioprotective effects of tyrosol. Tyrosol stimulated resistance to oxidative stress and also has anti aging effect.

Sudjaroen et al also reported quantitative results on catechin and epicatechin content of Tamarind seed and pericarp from Thailand [19]. Although catechin content of *Pinus eldarica* is two times higher than Tamarind pericarp, its epicatechin content is less (0.01% vs. 0.02%,). Such difference could not be attributed to differences in the measurement technique since both studies determined polyphenols using HPLC method.

Climate is a potential factor which affects the polyphenol content of pine tree. Exposure to light, soil, ripeness and stress are affected by climate variations [20, 21]. However the difference in the polyphenol content between our study and the mentioned Turkish study [9] could not be justified by seasonal variation; because in both studies the samples were collected during the summer season.

The catechin and epicatechin content of Pinus eldarica is comparable with those in edible food sources. In a review of food sources by Manach et al., chocolate had the highest amount of catechins and epicatechins (920 - 1220 ppm) [22]. This amount was 725.76 ppm in the bark of *Pinus eldarica*. On the other hand, the catechin and epicatechin content of Pinus eldarica bark is higher than that of other food sources such as green tea (50 - 400 ppm), beans (170 - 275 ppm), blackberry (130 ppm), apricot (50 -125 ppm), cherry (25 - 110 ppm), grape (15 - 87.5 ppm), peach (25 - 70 ppm) and apple (10-60 ppm).

Catechin and epicatechin are forms of polyhydroxy flavan-3-ol which are found in high concentrations in pycnogenol which is an extract of maritime pine. Several studies have shown beneficial effects of pycnogenol for health mainly due to its antioxidant properties and consequent excellent free radical scavenging function [13, 23]. Such antioxidant properties are potentially protective against cardiovascular disease [24, 25], based on our knowledge, tyrosol has not previously been detected in pycnogenol. However, the tyrosol proportion of Pinus eldarica is considerable, which could be a privilege for this species of *Pinus eldarica* could be a useful tool for nutrition therapy due to its beneficial health effect

Regarding the fact that *P. eldarica* belongs to the whole family of pines which contains appreciable amounts of polyphenols in its bark with potential antioxidant properties and also a significant amount of tyrosol in Pinus eldarica and health - protective effect of tyrosol, we suggest further studies and clinical trials concerning various health benefits of *P. eldarica* bark extract in the future.

Conclusion

Touch upon the fact that phenolic compounds of *P. eldarica* show significant antioxidant activity through various mechanisms [26], and the high amount of total phenolic compounds in its bark; This might be attractive for studying their health benefits through designing clinical trials.

It is recommended to measure the same polyphenols in different sub-species of Pinus eldarica in future studies. Moreover, sample collection both in summer and winter is

desirable in order to compare seasonal variations in the range of polyphenols in this same species of pine tree.

Acknowledgement

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Conflict of interest

The authors declare that they have no conflict of interest.

References

1. Facino RM, Carini M, Aldini G, Berti F, Rossoni G, Bombardelli E and Morrazoni P. Diet enriched with procyanidins enhances antioxidant activity and reduces myocardial post-ischaemic damage in rats. *Life Sci*. 1999; 64: 627 – 42.

2. Bagchi D, Bagchi M, Stohs SJ, Das D.K, Ray SD, Kuszynski CA et al. Free radicals and grape seed proanthocyanidin extract: importance in human health and disease prevention. *Toxicol.* 2000; 148: 187 – 97.

3. Lu Y, Zhao WZ, Chang Z, Chen WX and Li L. Procyanidins from grape seeds protect against phorbol ester-induced oxidative cellular and genotoxic damage. *Acta Pharmacol. Sin.* 2004; 25: 1083 – 9.

4. Sugisawa A, Inoue S and Umegaki K. Grape seed extract prevents H(2)O(2)-induced chromosomal damage in human lymphoblastoid cells. *Biol. Pharm. Bull.* 2004; 27: 1459 – 61.

5. Sato M, Yamada Y, Matsuoka H, Nakashima S, Kamiya T, Ikeguchi M and Imaizumi K. Dietary pine bark extract induces atherosclerotic lesion development in male Apo-E-deficient mice by lowering the serum cholesterol level. *Biosci. Biotechnol. Biochem.* 2009; 73: 1314 - 7.

6. Kamuren ZT, McPeek RA and Watkins JB. Effects of low-carbohydrate diet and Pycnogenol treatment on retinal antioxidant enzymes in normal and diabetic rats. *J. Ocul. Pharmacol. Ther.* 2006; 22: 10 - 18.

7. Maritim A, Dene BA, Sanders RA and Watkins JB. Effects of Pycnogenol treatment on oxidative stress in streptozotocin-induced diabetic rats. *J. Biochem. Mol. Toxicol.* 2003; 17: 193 - 9.

8. Farid R, Mirfeizi Z, Mirheidari M, Rezaieyazdi Z, Mansouri H, Esmaeli H, Zibadi S, Rohdewald P and Watson RR. Pycnogenol supplementation reduces pain and stiffness and improves physical function in adults with knee osteoarthiritis. *Nutr. Res.* 2007; 27: 692 - 7.

9. Yesli-Celiktas O, Ganzera M, Akgun I, Sevimli C, Korkmaz KS and Bedir E. Determination of polyphenolic constituents and biological activities of bark extracts from different Pinus species. *J. the Science of Food and Agriculture* 2009; 89 (8): 1339 - 45.

10. Pasqualini V, Robles C, Garzino S, Greff S, Bousquet- Melou A and Bonin G. Phenolic

compounds in *Pinus halepensis* Mill. Needles. a bioindicator of air pollution. *J. Chemosphere* 2003; 52: 239 - 48.

11. Cannac M, Pasqualini V, Greff S, Fernandez C and Ferrat L. Characterization of Phenolic Compounds in Pinus laricio Needles and Their Responses to Prescribed Burnings. *Molecules* 2007; 12: 1614 - 22.

12. Kanerva S, Kitunen V, Loponen J and Smolander A. Phenolic compounds and terpenes in soil organic horizon layers under silver birch, Norway spruce and Scots pine. *Biol. Fertil. Soils* 2008; 44: 547 – 56.

13. Rohdewald P. A review of the French matitime pine bark extract (Pycnogenol®), a herbal medication with a diverse pharmacology. *Int. J. Clin. Pharmacol. Ther.* 2002; 40: 158 – 68.

14. Kollesch J and Nickel D. Antike Heilkunst. Ausgewa¨hlte Texte aus den medizinischen Schriften der Griechen und Ro¨mer. Stuttgart: *Reclam*. 1994; 299 - 300.

15. Masquelier J. plan extract with proanthocyanidins content as therapeutic agent having radical scavenger effect and use therof. *Int. J. Vit. Nat. Res.* 1979; 49: 307 - 11.

16. Boros B, Jakabova S, Dornyei A, Gyorgyi H, Zsuzsanna P, Kilar F, et al. Determination of polyphenolic compounds by liquid chromatography-mass spectrometry in Thymus species. *J. Chromatography* 2010; 1217: 7972 - 80.

17. Pfundestein B, Desouky SE, Hull WE, Haubner R, Erben G and Owen RW. Polyphenol compounds in the fruits of Egyptian medicinal plants (Terminalia, bellerica, Terminalia chebula and Termialia horrida): Characterization, quantitation and determination of antioxidant capacities. *Phytochem.* 2010; 71: 1132 - 48.

18. Kilic A, Hafizoglu H, Tümen I, Dönmez IE, Sivrikaya H and Hemming J. Phenolic extractives of cones and berries from Turkish coniferous species. *Eur. J. Wood Prod.* 2011; 69: 63 – 6.

19. Sudjaroen Y, Haubner R, Wurtele G, Hull WE, Erben G, Spiegelhalder B, Changbumrung S, Bartsch H and Owen RW. Isolation and structure elucidation of phenolic antioxidants from Tamarind (*Tamarindus indica* L.) seeds and pericarp. *J. Food and Chemical. Toxicol.* 2005; 43: 1673 - 82.

20. Macheix J-J, Fleuriet A and Billot J**.** Fruit phenolics. Boca Raton, FL: CRC Press; 1990.

21. Parr AJ and Bolwell GP. Phenols in the plant and in man. The potential for possible nutritional enhancement of the diet by modifying the phenol content or profile. *J. Agric. Food Chem.* 2000; 80: 985 – 1012.

22. Manach C, Scalbert A, Morand C, Rémésy C, Jiménez L. May. Polyphenols: food sources and bioavailability. *Am. J. Clin. Nutr*. 2004; 79 (5): 727 - 47.

23. Cossins E, Lee R and Packer L. ESR Studies of Vitamin C Regeneration, Order of Reactivity of Natural Source Phytochemical Preparations. *Biochem. Mol. Biol.* Int 1998; 45: 583 – 98.

24. Saliou C, Rimbach G, Moini H, McLaughlin L, Hosseini S, Lee J, Watson RR and Packer L. Solar Ultraviolet-Induced Erythema in Human Skin and Nuclear factor-kappa-B-dependent Gene Expression in Keratinocytes Are Modulated by a French Maritime Pine Bark Extract. *Free Radic. Biol. Med*. 2001; 30: 154 – 60.

25. Spadea L and Balestrazzi E. Treatment of Vascular Retinopathy with Pycnogenol®. *Phytother. Res*. 2001; 15: 219 – 23.

26. Shahidi F, Amarowicz R, He Y H *et al*. Antioxidant activity of phenolic extracts of evening primrose (*Oenothera biennis*). A preliminary study. *J. Food Lip*. 1997; 4: 75 - 86.