Research in Pharmaceutical Sciences, August 2014; 9(4): 243-250
Received: Apr 2013
Accepted: Jul 2013

School of Pharmacy & Pharmaceutical Sciences
Isfahan University of Medical Sciences

Original Article

Phytochemical analysis of *Pinus eldarica* bark

S. Iravani and B. Zolfaghari*

Department of Pharmacognosy and Isfahan Pharmaceutical Research Center, Faculty of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, I.R. Iran.

Abstract

Bark extract of *Pinus pinaster* contains numerous phenolic compounds such as catechins, taxifolin, and phenolic acids. These compounds have received considerable attentions because of their anti-inflammatory, antimutagenic, anticarcinogenic, antimetastatic and high antioxidant activities. Although *P. pinaster* bark has been intensely investigated in the past; there is comparably less information available in the literature in regard to *P. eldarica* bark. Therefore, the aim of this study was to determine the chemical composition of *P. eldarica* commonly found in Iran. A reversed-phase high pressure liquid chromatography (RP-HPLC) method for the determination of catechin, caffeic acid, ferulic acid, and taxifolin in *P. pinaster* and *P. eldarica* was developed. A mixture of 0.1% formic acid in deionized water and 0.1% formic acid in acetonitrile was used as the mobile phase, and chromatographic separation was achieved on a Nova pack C18 at 280 nm. The two studied Pinus species contained high amounts of polyphenolic compounds. Among four marker compounds, the main substances identified in *P. pinaster* and *P. eldarica* were taxifolin and catechin, respectively. Furthermore, the composition of the bark oil of *P. eldarica* obtained by hydrodistillation was analyzed by gas chromatography/mass spectroscopy (GC/MS). Thirty-three compounds accounting for 95.1 % of the oil were identified. The oils consisted mainly of mono- and sesquiterpenoid fractions, especially α-pinene (24.6%), caryophyllene oxide (14.0%), δ-3-carene (10.7%), (E)-β-caryophyllene (7.9%), and myrtenal (3.1%).

Keywords: *P. pinaster; P. eldarica;* HPLC; GC/MS; Pine bark extract

INTRODUCTION

Nowadays, there has been an intense scientific interest in discovering new natural antioxidant agents (1,2). *Pinus pinaster* bark extract has been used worldwide as herbal remedy and nutrition supplemental food in many kinds of chronic and degenerative diseases (3,4). It contains numerous phenolic compounds such as catechins, taxifolin, and phenolic acids. The structures of catechin, caffeic acid, ferulic acid, and taxifolin are shown in Fig. 1. These compounds have received considerable attentions because of their anti-inflammatory, antimutagenic, anticarcinogenic, antimetastatic and high antioxidant activities (3-5). Several researches indicated the pharmaceutical and nutriceutical effects of polyphenolic components investigated in this study (3-5). For instance, catechins might be useful in body fat and malondialdehyde-modified low density lipoprotein (LDL) reduction, and in the prevention and improvement of other lifestyle-related diseases (6). Green tea catechins might have potential to inhibit the endonuclease activity of influenza A virus RNA polymerase (7).

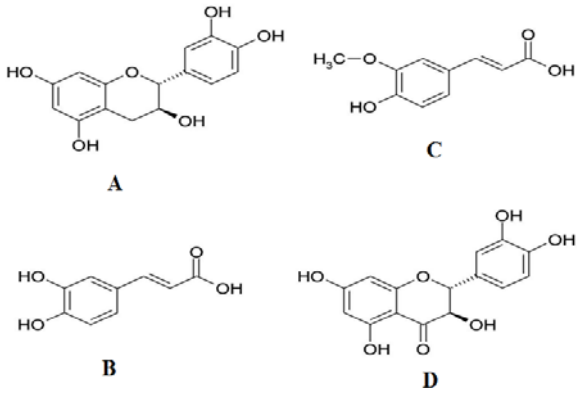

Fig. 1. Structures of phenolic compounds detected in pine bark extracts. A; catechin, B; caffeic acid, C; ferulic acid, D; taxifolin.

*Corresponding author: B. Zolfaghari, this paper is extracted from the Pharm.D thesis No. 388288.
Tel: 0098 311 7922648, Fax: 0098 311 6680011
E-mail address: zolfaghari@pharm.mui.ac.ir

It was also reported that polyphenolic compounds including catechins, ((-)-epigallocatechin gallate, (-)-epicatechin gallate and (-)-epigallocatechin from green tea had antiviral effect against influenza virus. This effect is mediated not only by specific interaction with HA protein, but altering the physical properties of viral membrane (8). Taxifolin was found to inhibit the synthesis and secretion of triacylglycerol and phospholipids, and inhibit cholesterol synthesis in a dose- and time-dependent manner. It also suppressed HMG-CoA reductase activity and cholesteryl ester formation, down-regulated the expression of intercellular adhesion molecule-1 (ICAM-1), and decreased the secretion of apoB into LDL-like particles (9-11). Furthermore, the identified phenolic acids (ferulic and caffeic acids) have antioxidants activity against reactive oxygen radicals which cause damage to cell membranes and DNA. These components might be useful in prevention of cancers, cardiovascular disorders, and neurodegenerative diseases (1,3).

Pinus pinaster (Pinaceae) is a tree of 20-35 m tall (medium-sized tree); the bark is orange-red, thick and deeply fissured at the base of the trunk, somewhat thinner in the upper crown. The needles are in pairs, 12-22 cm long and bluish-green to distinctly yellowish-green. The cones, 10-20 cm long and 4-6 cm broad at the base when closed, are green at first, ripening to glossy red-brown when 24 months old.

There are more than 4 million hectares of *P. pinaster* forest all around the occidental Mediterranean basin (Portugal, Spain, France, Italy, Tunisia, Algeria and Morocco). *P. pinaster* also grows in the north of Iran (especially Nowshahr, Chaloos, and Kiashahr). This pine is especially exploited for its wood and resin products. The use of pine needles to produce the essential oil is another possibility for integral profit of pine forests (12,13). *P. eldarica* is one of the most common pines in Iran. It is a medium-sized tree, reaching 12-15 m high. The bark is brownish-gray or light gray, not flaking; head broad-topped. The leaves are stiff, 6-9 cm long and green. The cones are pedunculate, solitary or in pairs and light reddish-brown. Scales irregularly rhombic, glossy, smooth, the whitish-gray apophysis concave: seeds blackish, 6-7 mm long, the reddish-brown wing 18-28 mm long (14). Although *P. pinaster* bark has been intensely investigated in the past, there is comparably less information available in the literature in regard to *P. eldarica* bark. Therefore, the aim of this study was to determine the chemical composition of *P. eldarica* commonly found in Iran.

MATERIALS AND METHODS

Oil preparation

Pinus eldarica powders (100 g) were hydro-distilled (with 1.2 liter of water) in a Clevenger-type apparatus for 4 h according to British Pharmacopoeia (15). The volatile oil was collected with pentane and dried over anhydrous sodium sulfate and stored in sealed vial at 4 °C until analysis. The yield of oil was calculated based on dried weight of plant material. Pale yellow oil from the bark was obtained (0.15 % v/w).

Gas chromatography/mass spectroscopy analysis

GC-MSanalysis was performed on a Hewlett Packard 5792A mass selective detector coupled with a Hewlett Packard 6890 gas chromatograph, equipped with a HP-5MS capillary column (30 m × 0.25 mm, film thickness 0.25 μm). The GC operating conditions were as follows: carrier gas; helium with a flow rate of 2 mL/min, column temperature; 60-280 °C at 4 °C/min, injector and detector temperatures; 280 °C, volume injected; 0.1 mL of the oil, split ratio; 1:50. The MS operating parameters were as follows: ionization potential; 70 eV, ion source temperature; 250 °C, resolution; 1000, ionization current; 750 μA, mass range; 35-425.

Identification of the components of the essential oils

Identification of the constituents was based on computer matching against the library spectra (Library Database Wiley 275L), their retention indices with reference to an n-alkane series in a temperature programmed run, interpreting their fragmentation pattern and comparison of the mass spectra with those reported in the literatures (16,17).

High pressure liquid chromatography analysis

Reagents

The reagents were as follows: a; H_2O-deionized high pressure liquid chromatography (HPLC) water, b; acetonitrile (Merck, for liquid chromatography), c; methanol (Merck, for liquid chromatography), d; formic acid (Merck), e; chemical reference standards containing catechin (≥98% HPLC), caffeic acid (≥99.0% HPLC), ferulic acid (≥99.0% HPLC), and taxifolin (≥98% HPLC) all from Sigma Aldrich, f; HPLC mobile phase which mobile phase A was 0.1 % formic acid in H_2O, and mobile phase B was 0.1 % formic acid in acetonitrile.

Plant material

Pine bark specimens were collected from two different locations in Iran: *P. pinaster* from Nowshahr/Chaloos (36°39′11.47″N 51°29′56.93″E / 36°39'22"N 51°25'40"E) and *P. eldarica* from Isfahan (32°38′N 51°39′E, altitude, 1590 m). The samples were collected between August and September 2009. The specimens were dried at room temperature, ground by using a conventional grinder and stored at +4 °C.

Preparation of pine bark extracts

Pine bark extract was obtained by the method developed by Masquelier (18). Pine bark (100 g) was ground for 1 min, at a speed setting of 2 using a mixer to obtain coarse powder, extracted with 600 mL of boiling water, and then cooled down to 20 ºC.

After filtration, 250 mL of liquid were collected and sodium chloride was added up to saturation, and the precipitate formed was removed by filtration. Subsequently, the filtrate was extracted three times with ethyl acetate (10 mL filtrate per 1 mL ethyl acetate (v/v)).

The ethyl acetate phase was collected and dried using anhydrous sodium sulfate and reduced to 1/5 of its volume in a rotary vacuum evaporator. The extract was then poured into three volumes of chloroform, while stirring mechanically. The proanthocyadins were precipitated and collected by filtration. The light beige color powder obtained was stored at −20 ºC. All chemicals were of analytical grade purity.

Standard preparation

Stock solutions of catechin, caffeic acid, ferulic acid and taxifolin were prepared in methanol (1 mg/ml) which were stable for a week in the dark and at 0 °C. The stock solutions were used for working standards (10, 20, 60, 80, and 100 µg/ml) and standard calibration curves. All the standards were injected at different times (n value=9).

HPLC conditions

The HPLC system consisted of a manual injector, a HPLC pump (515 Waters), a C18 Nova pack reversed-phase- column (3.9 × 150 mm, 4 µm), a spectrophotometer UV-Vis detector (Dual absorbance, 2448 Waters) controlled by computer software (Waters Empower).

The HPLC gradient profile comprising A; 0.1% formic acid in H_2O and B; 0.1% formic acid in acetonitrile , was as follows: 0-40 min; linear gradient from 92:8 (v/v) to 34:66 (v/v), 40-45 min; linear gradient from 34:66 (v/v) to 98:2 (v/v, column rinsing), 45-50 min; 98:2 isocratic, 50-52 min; 98:2 (v/v) to 92:8 (v/v), 52-57; 92:8 (v/v) isocratic. The flow rate was 1.0 ml/min and the analysis was carried out at room temperature. The wavelength for UV detection was set at 280 nm, and the injection volumes were 20 µL.

Accuracy

Accuracy was measured as the percent of deviation from the nominal concentration. Accuracy solutions of catechin, caffeic acid, ferulic acid, and taxifolin (10, 20, 60, 80, and 100 µg/ml) were accurately prepared and injected to the HPLC in triplicate. The response was used for back calculation of concentration according to the calibration curve equation. The back calculated concentration was compared to the nominal concentration and the percent deviation was calculated.

Statistical analysis

The data were processed using Microsoft Excel for averages and standard deviation. Data are presented as mean values ± SD.

RESULTS

HPLC analysis

Sample solutions were prepared in methanol, and analyzed by HPLC assay (19). We noticed that determination at 280 nm was much more suitable (19). Four components (catechin, caffeic acid, ferulic acid, and taxifolin) were identified in HPLC chromatograms of *P. pinaster* and *P. eldarica* samples based on the retention time by comparison with retention time of reference standard (Figs 2 and 3). The individual signals were assigned by addition of 30 micrograms of each standard (catechin, caffeic acid, ferulic acid, and taxifolin) into *P. pinaster* sample (Fig. 4). In order to obtain quantitative results, the standard of the four identified compounds were used to establish calibration curves. The correlation between the peak area ratios and the standard concentrations was evaluated over the range 10-100 µg/ml, and was found to be linear (Table 1).

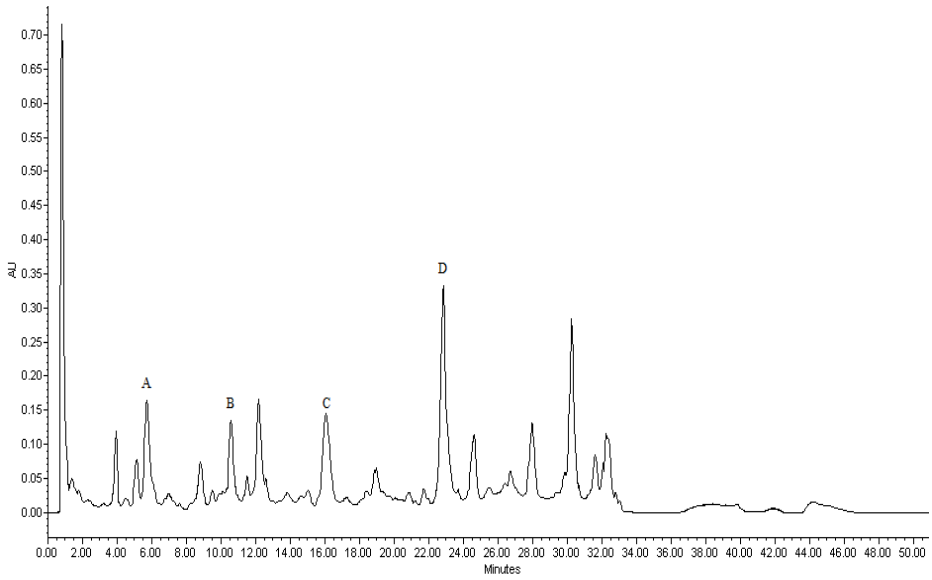

Fig. 2. HPLC chromatogram of *P. pinaster* sample investigated. A; catechin, B; caffeic acid, C; ferulic acid, D; taxifolin.

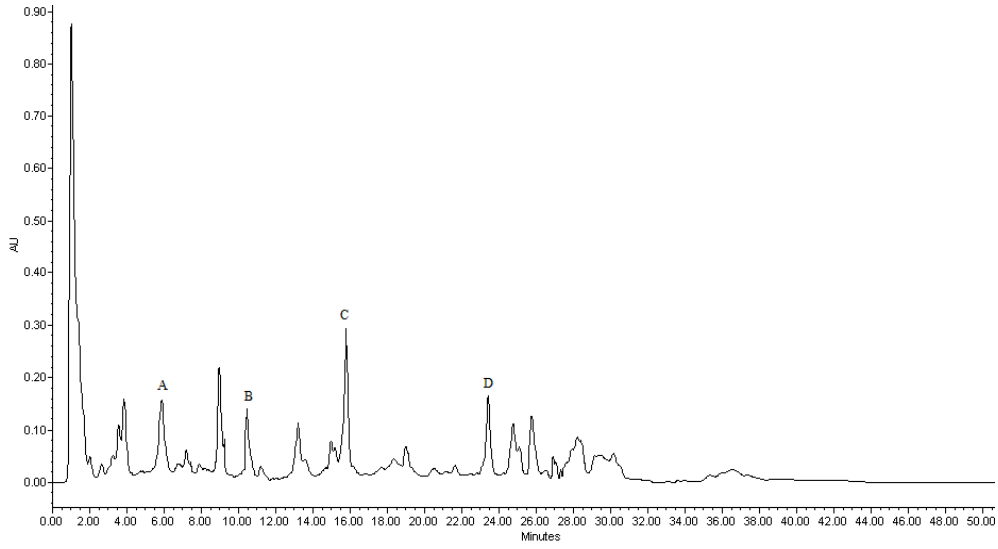

Fig. 3. HPLC chromatogram of *P. eldarica* sample investigated. A; catechin, B; caffeic acid, C; ferulic acid, D; taxifolin.

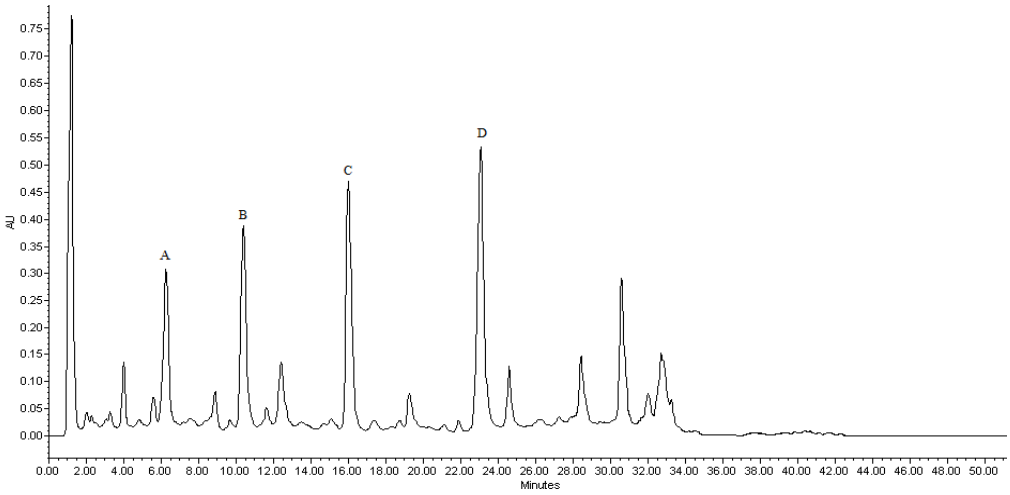

Fig. 4. HPLC chromatogram of standards. A; catechin, B; caffeic acid, C; ferulic acid, D; taxifolin.

Table 1. Linear regression analysis data of catechin, caffeic acid, ferulic acid, and taxifolin (n value = 9).

Phenolic compounds	Calibration range (µg/ml)	R^2	%Y-Intercept
Catechin	10-100	0.9978	0.98
Caffeic acid	10-100	0.9981	0.16
Ferulic acid	10-100	0.9987	1.58
Taxifolin	10-100	0.9985	0.64

Table 2. Quantitative results for *P. pinaster* and *P. eldarica* (percentage of each compound in the respective pine bark extract; Mean ± SD; n=3).

Phenolic compounds	*P. pinaster*	*P. eldarica*
Catechin	4.12 ± 0.19	3.41 ± 0.22
Caffeic	1.92 ± 0.12	1.62 ± 0.07
Ferulic	2.33 ± 0.09	2.27 ± 0.21
Taxifolin	6.68 ± 0.32	1.95 ± 0.08

Table 3. Evaluation of accuracy of the proposed method for determination of catechin, caffeic acid, ferulic acid, and taxifolin (n=3).

Phenolic compounds	Nominal concentration (µg/ml)	Real concentration (µg/ml)			
		Mean	SD	RSD	% Recovery
Catechin	10	9.375	0.445	4.746	93.75
	20	18.75	0.611	3.258	93.76
	60	61.69	3.614	5.858	102.8
	80	77.25	6.239	8.076	96.57
	100	101.4	5.017	4.943	101.4
Caffeic acid	10	9.592	0.486	5.067	95.92
	20	20.38	0.595	2.918	101.9
	60	61.98	3.217	5.190	103.3
	80	77.50	4.950	6.387	96.88
	100	100.7	6.699	6.648	100.7
Ferulic acid	10	8.923	0.576	6.455	89.23
	20	18.91	1.488	7.866	94.59
	60	59.91	3.582	5.978	99.86
	80	77.82	4.644	5.967	97.28
	100	102.1	5.315	5.204	102.1
Taxifolin	10	10.39	0.879	8.458	103.9
	20	19.52	2.005	10.27	97.61
	60	57.22	4.874	8.519	95.38
	80	81.04	4.772	5.890	101.3
	100	100.8	3.412	3.382	100.8

Table 4. Percentage composition of the bark oil of the *P. eldarica* Medw.

Number	Retention Time	Compound	% (from TIC data)	Retention Indices
1	3.835	α-pinene	24.6	939
2	4.081	camphene	1.1	952
3	4.173	verbenene	1.3	957
4	4.488	1,3,5-cycloheptatrine, 3,7,7-trimethyl	2.2	973
5	4.619	β-pinene	0.7	979
6	4.951	mesitylene	0.7	994
7	5.340	δ-3-carene	10.7	1012
8	5.598	p-cymene	0.3	1023
9	5.661	m-cymene	1.9	1026
10	5.758	limonene	1.5	1030
11	6.507	γ-terpinene	0.4	1060
12	7.125	2-p-tolypropene	0.6	1082
13	7.308	o-allyltoluene	1.8	1088
14	8.361	α-campholenal	1.7	1126
15	8.716	trans-pinocarveol	2.4	1138
16	8.899	camphor	0.4	1145
17	9.008	α -phellandren-8-ol	0.5	1149
18	9.363	isopinocamphone	0.7	1160
19	9.426	pinocarvone	0.9	1162
20	9.551	borneol	1.6	1166
21	9.866	4-carvomenthenol (4-terpineol)	0.7	1176
22	10.278	α-terpineol	1.1	1188
23	10.450	Myrtenal	3.1	1193
24	10.856	verbenone	0.7	1206
25	12.378	cis-myrtanol	0.7	1257
26	16.864	longifolene	0.5	1399
27	17.339	(E)-β-caryophyllene	7.9	1414
28	18.346	-selineneβ	2.0	1449
29	22.168	caryophyllene oxide	14.0	1578
30	22.872	endo-2-methylbicyclo[3.3.1] nonane	2.1	1600
31	23.628	*caryophylia-4(12)-8(13)-dien-5*-beta-ol	2.1	1629
32	24.600	cyclohexane,1,5-diethenyl-3-methyl-2 methylene	3.8	1664
33	32.611	(z)-myrtanyl acetate	0.4	1984

The four polyphenolic compounds content of *P. pinaster* and *P. eldarica* bark extracts are shown in Table 2. The extraction yield for *P. pinaster* is 20% and for *P. eldarica* is 22%. The accuracy of the developed method was evaluated by back calculation method. The results are expressed as percent recoveries of catechin, caffeic acid, ferulic acid, and taxifolin in the samples (Table 3). As shown in table 3, the minimum of recovery was 89.23%. In higher concentrations of each compound, the recovery percent is higher than 100% which is due to some errors.

Essential oil composition of the bark of Pinus eldarica Medw.

Thirty-three constituents, representing 95.1 % of the bark oil were identified (Table 4). α-pinene (24.6 %), δ-3-carene (10.7 %), and myrtenal (3.1 %) were the main constituents of the monoterpenoid fraction of the bark oil, while (E)-β-caryophyllene (7.9 %) and caryophyllene oxide (14.0 %) were the main components of sesquiterpenoid fraction of the same oil. The composition of *P. eldarica* bark oil is characterized by high content of monoterpenes (62.2 %).

DISCUSSION

HPLC analysis

Among the four marker compounds investigated, the major substances identified in *P. eldarica* and *P. pinaster* bark extracts were catechin (3.414%) and taxifolin (6.68%), respectively. The total amounts of the four identified compounds in *P. pinaster* and *P. eldarica* bark extracts were 15.055% and

9.273%, respectively. Previous studies also reported that the main substances identified in *P. pinaster* bark extract were taxifolin, catechin, and ferulic acid (20,21). It was demonstrated that the herbal extracts that showed strong antioxidant activity, also possessing high amounts of phenolic compounds, especially catechins and taxifolin (22).

Yesil-Celiktas and co-workers analyzed the polyphenolic composition of four pine bark extracts (*P. brutia*, *P. nigra*, *P. sylvestris*, and *P. pinea*). They suggested that the other Pinus species like *P. brutia* which contained extremely high concentrations of taxifolin (approximately 18.5%) had high biological activities, and could be used for commercial applications instead of French *P. pinaster* (22).

GC/MS analysis

It was reported that the main constituents of the leaf oil of *P. eldarica* were germacrene D (26.6%), β-caryophyllene (17.1%), α-pinene (11.8%), β-pinene (7.9%), elemicin (4.3%), and α-humulene (4.2%). Main components of the fruit oil were β-caryophyllene (34.0%), α-pinene (16.3%), longifolene (10.5%), α-humulene (6.4%), δ-3-carene (6.3%), and β-pinene (3.8%) (23).

The compounds of turpentine oil from Russian *P. eldarica* are relatively stable, and the oil content of the resin has been reported to be 15% to 19%. The main components of Monoterpenes were α-pinene (31.5% to 64.2%), δ-3-carene (16.9% to 42.8%), and β-pinene (2.5% to 11.7%) (24).

In another study, the major components of the monoterpene and sesquiterpene fractions of the oil obtained from the terpenoid resin of *P. eldarica* were α-pinene and β-caryophyllene (25). Furthermore, we analyzed the composition of essential oil obtained from the bark of *P. pinaster* (26).

As a result, the composition of the oil is characterized by high content of monoterpenes (70.6%). α-pinene (63.9%) was the main constituent of the monoterpenoid fraction while junipene (7.5%) and β-caryophyllene (14.3%) were the main components of sesquiterpenoid fraction of the bark oil (26).

CONCLUSION

This study shows that RP-HPLC method enables the identification and quantification of catechin, caffeic acid, ferulic acid, and taxifolin in *P.eldarica* and *P. pinaster* bark extracts. Previous studies introduced *P. pinaster* as a source of polyphenolic compounds. Our results demonstrated that *P. eldarica* bark extracts can be used as an effective source of polyphenolic compounds in the food and pharmaceutical industries, as well. Furthermore, because of the high percentage of α-pinene in the volatile oils obtained from the bark of *P. eldarica*, it can be used in industries and especially in production of plasticizers, camphor, insecticides, perfumes, and solvents.

ACKNOWLEDGMENTS

This study was a part of the Pharm.D thesis of Pharmacognosy department, Faculty of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences (Registration No. 388288).

REFERENCES

1. Packer L, Rimbach G, Virgili F. Antioxidant activity and biologic properties of a procyanidin-rich extract from the pine (*Pinus maritima*) bark, Pycnogenol. Free Rad Biol Med. 1999;27:704-724.
2. Grimm T, Schafer A, Hogger P. Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract (Pycnogenol). Free Rad Biol Med. 2004;36:811-822.
3. Rohdewald P. A review of the French maritime pine bark extract (Pycnogenol), a herbal medication with a diverse clinical pharmacology. Int J Clin Pharmacol Ther. 2002;40:158-168.
4. Iravani S, Zolfaghari B. Pharmaceutical and nutraceutical effects of *Pinus pinaster* bark extract. Res Pharm Sci. 2011;6:1–11.
5. Blazso G, Gabor M, Sibbel R, Rohdewald P. An anti-inflammatory and superoxide radical scavenging activities of a procyanidins containing extract from the bark of *Pinus pinaster* sol. and its fractions. Pharm Parmacol Lett. 1994;3:217-220.
6. Nagao T, Komine Y, Soga S, Meguro S, Hase T, Tanaka Y, *et al*. Ingestion of a tea rich in catechins leads to a reduction in body fat and malondialdehyde-modified LDL in men. Am J Clin Nutr. 2005;81:122-129.

7. Kuzuhara T, Iwai Y, Takahashi H, Hatakeyama D, Echigo N. Green tea catechins inhibit the endonuclease activity of influenza A virus RNA polymerase. PLoS Curr. 2009;1:RRN1052
8. Song JM, Lee KH, Seong BL. Antiviral effect of catechins in green tea on influenza virus. Antiviral Res. 2005;68:66-74.
9. Krecman V, Skottova N, Walterova D, Ulrichova J, Simanek V. Silymarin inhibits the development of diet-induced hypercholesterolemia in rats. Planta Med. 1998;64:138-142.
10. Casaschi A, Rubio BK, Maiyoh GK, Theriault AG. Inhibitory activity of diacylglycerol acyltransferase (DGAT) and microsomal triglyceride transfer protein (MTP) by the flavonoid, taxifolin, in HepG2 cells: potential role in the regulation of apolipoprotein B secretion. Atherosclerosis. 2004;176:247-253.
11. Wang YH, Wang WY, Liao JF, Chen CF, Hou YC, Liou KT, *et al*. Prevention of macrophage adhesion molecule-1 (Mac-1)-dependent neutrophil firm adhesion by taxifolin through impairment of protein kinase-dependent NADPH oxidase activation and antagonism of G protein-mediated calcium influx. Biochem Pharmacol. 2004;67:2251-2262.
12. Chagné D, Brown G, Lalanne C, Madur D, Pot D, Neale D, *et al*. Comparative genome and QTL mapping between maritime and loblolly pines. Mol Breed. 2003;12:185–195.
13. Ribeiro MM, Plomion C, Petit R, Vendramin GG, Szmidt AE. Variation in chloroplast single-sequence repeats in Portuguese maritime pine (*Pinus pinaster* Ait.). Theor Appl Genet. 2001;102:97-103.
14. Parsa A. flora of iran. sponsord by ministry of science & higher. Education of iran, Tehran. 1978;1:448-449. .
15. British Pharmacopoeia. (1998). Vol. 2. HMSO, London. A137-A138.
16. Adams RP. Identification of essential oil components by gas chromatography/mass spectroscopy. Illinois: Allured Publishing Corporation; 1995. p. 69-351.
17. Swigar AA, Silverstein RM. Monoterpenes. infrared, mass, 1H-NMR, 13C-NMR spectra and kovats indices. Wisconsin: Aldrich Chemical Company; 1981. p. 1-121.
18. Masquelier J. Plant extract with proanthocyanidins content as therapeutic agent having radical scavenger effect and use thereof. US Patent 4,698,360, 1987.
19. Chen P, Song F, LZ L. Chromatographic fingerprint analysis of Pycnogenol dietary supplements. J AOAC Int. 2009;92:624-632.
20. Sarikaki V, Rallis M, Tanojo H, Panten I, Dotsiva Y, Laukas YL. In vitro percutaneous absorption of pine bark extract (PYC) in human skin. J Toxicol Cutaneous Ocular Toxicol. 2004;23:149–158.
21. Romani A, Ieri F, Turchetti B, Mulinacci N, FF V, Buzzini P. Analysis of condensed and hydrolysable tannins from commercial plant extracts. J Pharm Biomed Anal. 2006;41:415–420.
22. Yesil-Celiktas O, Ganzera M, Akgun I, Sevimli C, KS K, Erdal B. Determination of polyphenolic constituents and biological activities of bark extracts from different Pinus species J Sci Food Agric. 2009;89:1339–1345.
23. Afsharypour S, San'aty F. Essential oil constituents of leaves and fruits of *Pinus eldarica* Medw. L. J Essent Oil Res. 2005;17:327-328.
24. Chundnyi AV, Rudenko B-A. Composition of turpentine oil from *P. eldarica* Medw. Rastit Resur. 1982;18:252-255.
25. Drebushchack TD, Shmidt EN, Khan VA, Dubovenko ZV, Kemertelidze EP, Pentegova VA. Terpenoids of oleoresins of *Pinus pityusa* and *P. eldarica*. Chem Natural Comp. 1982;18:316-320.
26. Zolfaghari B, Iravani S. Essential oil constituents of the bark of *Pinus pinaster* from Iran J essent oil-bearing plants. 2012;15:348-351.