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Greek Fir Seeds and Cones as Underestimated Source of Essential Oil: Composition and Biological Properties

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Abstract: Greek fir (*Abies cephalonica*) seeds and cone scales were used, for the first time, for an analysis of their biologically active volatile compounds. It was observed that the yield of seed essential oil was 18%, which, among plants, is impressive. The seed essential oil was characterized by a distinctive forest scent with a subtle lemon undertone. The determination of the chemical compositions of the hydrodistilled oils isolated from the seeds and cone scales, achieved via chromatographic and spectroscopic methods (MS, NMR), revealed more than 100 compounds, mostly monoterpene hydrocarbons. The above methods allowed for the identification of 99.4% and 98.3% of the total seed and cone scale oil compositions, respectively. Limonene in its enantiomeric levorotatory form (*S*) constituted over 80% of the essential oil isolated from the *Abies cephalonica* seeds. This abundance of limonene makes them a potential natural source for obtaining this compound, which has demonstrated various biological properties. The main cone essential oil compounds were α- and β-pinenes as well as limonene. The cytotoxic effects of both essential oils were analyzed using the MTT assay in skin fibroblasts, keratinocytes, and melanoma cell lines, in the range of 0.012–0.2 µL/mL of essential oil. The cone scale essential oil was slightly more cytotoxic and induced a decrease in the cell viability in concentrations of 0.05–0.1 µL/mL, with small differences between the cell lines. The tested essential oils did not have selective effects on the melanoma cells (A375 and C32) when compared with normal cells. Both the seed and cone scale essential oils revealed good antimicrobial effects on *Staphylococcus aureus* and *Escherichia coli*.

Keywords: *Abies cephalonica*; essential oil; seed; cone; cytotoxic assay; antimicrobial agent

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1. Introduction

Greek fir, scientifically known as *Abies cephalonica* Loudon, is a species of fir tree native to southern Greece, specifically in the Peloponnese mountains, on Mt. Aenos on the island of Kefalonia, on Mt. Parnitha in Attica, and on the island of Euboea [1]. While Greek fir has been primarily valued for its timber [2], it also holds cultural and traditional significance in the region. The *Abies cephalonica* needles with short twigs can be used to isolate essential oil. Fir essential oil (EO) is known for its invigorating and uplifting scent. It is used in aromatherapy and the fragrance industry for its fresh and woody aroma [3]. Various parts of the Greek fir tree, including the bark and resin, have been used in traditional medicine [4]. The resin has been historically used for its healing properties and as an ingredient in pharmacology. Greek fir wood has been traditionally used for various purposes due to its durability, strength, and resistance to decay. It has been used in construction for beams, flooring, and furniture making. The timber is known for its attractive grain patterns and is sought after for decorative purposes [2]. Greek fir trees are often used as Christmas trees in Greece and other countries. These trees are cut and

decorated with ornaments and lights during the holiday season [2]. This species of fir tree is considered an important symbol of the natural heritage of Greece. It is often associated with the country's rugged mountains and is featured in folklore, art, and literature as a representation of strength, endurance, and beauty. In Poland, Greek fir grows much slower than in Greece or the Balkans. It is found only in gardens in Poland. The species prefers sun, moderately fertile, permeable, slightly moist soil, and moisture in the air, but it tolerates drought and heat well, which is very important in light of climate change [2,4,5].

The traditional use of *Abies cephalonica* EO is not as extensively documented as that of some other essential oils (EOs) [6,7]. However, like other fir EOs, it has been used in various ways for its aromatic and potential therapeutic properties. Greek fir EO (needle oil) is known for its fresh, woody, and invigorating scent. In aromatherapy, it has been used to promote a sense of grounding, mental clarity, and emotional balance. It is often used in diffusers and inhalers or added to massage oils and bath products to create a calming and uplifting atmosphere. Fir EOs have been used traditionally to support respiratory health. The inhalation of the EO or its diffusion may help to clear congestion, alleviate coughs, and promote easier breathing [8]. *Abies cephalonica* EO has been employed for its potential analgesic and anti-inflammatory properties. It may be used topically, diluted in a carrier oil, to massage sore muscles and joints, providing relief and relaxation. While the traditional use of Greek fir EO in skincare is not well documented, fir EOs, in general, are sometimes used in natural skincare products. They are believed to have cleansing, purifying, and toning effects on the skin. However, caution should be exercised, and a patch test is recommended, as EOs can cause skin irritation in some individuals. It is important to mention that traditional uses of EOs are based on historical practices and anecdotal evidence, and they may not have undergone extensive scientific research or regulatory approval. If the usage of Greek fir EO for any purpose is taken into consideration, it is advisable to consult with a qualified aromatherapist or healthcare professional to ensure safe and appropriate usage [9–11].

Abies cephalonica is a relatively underexplored fir species. Unlike a handful of studies that have examined the volatile compounds in its needles and bark [1,7,11–15], there has been a notable absence of research on the EOs found in intact seeds and cones. This gap in knowledge extends to both their chemical compositions and potential biological functions. Therefore, this study's main aim was to carry out a comparative analysis based on the contents, chemical profiles, and biological effects of EOs isolated from the seeds and cone scales of the Greek fir. The EOs were subjected to testing against pathogenic microbial strains: *Staphylococcus aureus* and *Escherichia coli*, which are the bacteria that most often cause skin infections. However, *Escherichia coli*, which is commonly found in the gut, may also be a causative agent when the epidermal barrier is damaged [16,17]. Notably, this is a pioneering investigation exploring the cytotoxic activities of fir EOs against melanoma cells and comparing them with those against dermal fibroblasts and keratinocytes. The ultimate aim was to evaluate the potential of using fir EOs as natural products in cosmetics.

2. Materials and Methods

2.1. Greek Fir Seeds and Cone Scales

Ripe cones were hand-picked from the upper crown of the *Abies cephalonica* in autumn 2012, and the analysis was performed in 2013. The tree is the property of the Polish Botanical Garden located in the middle of Poland (Lodz), the employees of which confirmed the species affiliation. The samples were stored in a freezer. The voucher specimens (nos. I-28/Acl/N/2012 and I-28/Acl/S/2012) of the seeds and cone scales were deposited at the Institute of Natural Products and Cosmetics, Lodz, Poland.

2.2. Parameters of Hydrodistillation

Hydrodistillation was preceded by the additional preparation of the raw material—seeds were husked from cones and ground in a mill. Fir EOs from cone scales and grounded seeds were isolated via hydrodistillation for 4 h in a Clevenger-type glass apparatus [18],

as was performed during similar experiments [19]. Hydrodistillations were performed 5 times in parallel. The hydrodistillation was also performed in the Clevenger apparatus (Farmakopea Polska VIII) for 4 h.

2.3. GC-MS Analysis of Essential Oils

The detection and quantification of the EO volatiles were performed using a Thermo Electron gas chromatograph (GC) coupled with a DSQ II quadrupole mass spectrometer (MS) and flame ionization detector (FID) (Thermo Fisher Scientific, Waltham, MA, USA). Simultaneous chromatographic analyses with the FID as a detector and spectroscopic scanning were performed based on an MS-FID splitter (SGE Analytical Science, Ringwood, Melbourne, VIC, Australia). MS spectra were recorded in the range of *m/z* 50–550, after electron impact ionization at 70 eV. Before analysis, the samples were dissolved (1:5) in a mixture of hexane:diethyl ether (1:1) (Poch Production, Gliwice, Poland). The injected samples (1 µL) were distributed in the split chamber, and the split ratio was 1:2–1:10. The chromatograph device was equipped with two columns. The first was a capillary, non-polar column: Restek Rtx-1 MS (60 m × 0.25 mm i.d.; film thickness: 0.25 µm) (Fisher Scientific, Loughborough, UK). The operating conditions were as follows: temp. program: 50 °C (3 min)–300 °C (30 min) at a rising rate temperature of 4 °C/min; the temperatures of the GC injector and GC detector were 280 °C and 300 °C, respectively, during analysis, as the carrier gas helium was used (constant pressure: 300 kPa). The second, enantioselective column was the Chirasil-Dex CB (Agilent, Santa Clara, CA, USA), the parameters of which were as follows: 30 m × 0.25 mm i.d.; 0.25 µm df; the enantiomers were separated at 50 °C (3 min)–220 °C (30 min) at a rate 4 °C/min; the injector was 240 °C and the detector was 250 °C; nitrogen, at a flow rate of 1.0 mL/min, was used as the carrier gas. In this column, the resolution of the levorotatory enantiomers and dextrorotatory enantiomers of the chosen monoterpene hydrocarbons (Sigma Aldrich, St. Louis, MO, USA) was performed. The area normalization method was used as the quantification method. The above methodology was used in previously published material [19].

2.4. Nuclear Magnetic Resonance Spectroscopy

^{1}H-NMR spectroscopy was performed using a Bruker Avance DPX 250 NMR Spectrometer (Bruker, Rheinstetten, Germany) at the Institute of Organic Chemistry, Lodz University of Technology, at an operating frequency of 250 MHz. $CDCl_3$ (99.95% for NMR, Merck Millipore, Burlington, MA, USA) and TMS (99%, Sigma-Aldrich) were used as the solvent and internal standard, respectively (Institute of Organic Chemistry, Lodz University of Technology).

2.5. Isolation of Volatiles

To increase the reliability of the analysis over the GC-MS, which sometimes gives ambiguous results, we tried to isolate pure compounds, and as many as possible. To isolate the selected volatiles, the seed EO (25.5 g) was fractional-distilled under reduced pressure. The rectification residue (1.63 g) was subjected to flash chromatography (FC) based on silica gel 60 (0.040–0.063 mm mesh, Merck) in a glass FC column of a 30 mm diameter, with, at first, *n*-hexane (Poch Production, Gliwice, Poland) as eluent, and then increasing amounts of diethyl ether (Poch Production, Gliwice, Poland), under the nitrogen as a propellant. The eluent was collected in 15 mL tubes. The separation was monitored via the thin-layer chromatography (TLC) method on a Kieselgel 60 aluminum plate (Merck). This methodology was also used in our previous experiments [19]. Seventeen fractions (1–17) were collected. All were analyzed using the above-described GC-MS method. Structures of 14 EO compounds were confirmed using ^{1}H-NMR spectroscopy: **1** (264 mg) (*E*)-β-caryophyllene (25.6%), longifolene (22.0%); **2** (110 mg) α-humulene (61.1%), β-elemene (10.1%); **6** (138 mg) α-terpinyl acetate (40.9%), dihydroagarofurane (19.9%); **7** (38 mg) (*E*,*E*)-farnesyl acetate (58.3%); **8** (208 mg) (*E*)-β-caryophyllene epoxide (38.7%); **9** (55 mg) (*E*,*E*)-farnesal (27.4%); **10** (25 mg) γ-eudesmol (28.1%), (*E*)-nerolidol (15.8%); **14** (101 mg)

(*E*,*E*)-farnesol (38.0%),β-elemol (14.0%). The masses of the fractions are presented in the first brackets, and the percentages of volatiles identified with the GC-FID detector are shown in the second brackets.

2.6. Determination of Essential Oil Composition

Identification of the EO compounds was achieved in a few ways, as cited before [19]. Firstly, a comparison of the collected mass spectra with spectra in the following mass libraries was performed: NIST98.1 and Wiley, 10th edition. Secondly, the relative retention indices of the experimental compounds on the non-polar column were compared with the literature RIs on the non-polar column. Thirdly, a comparison of the ^{1}H-NMR spectra of the isolated volatiles with literature data or a crafted NMR database (deposited at the Institute of Natural Products and Cosmetics, Lodz University of Technology) was performed. The identification of the enantiomers of the levorotatory and dextrorotatory forms of the monoterpene hydrocarbons α- and β-pinene, limonene, and camphene was based on the (−)- and (+)-standards of the above volatiles using a Chirasil-Dex CB column (Agilent, Santa Clara, CA, USA). To see details of gas chromatograms of EOs and fractions of seed EO as well NMR-spectra of seed EO fractions see the Supplementary Materials: Section S1.

2.7. Cell Viability Assay

Skin fibroblasts that were typical for healthy human (CCD25Sk) and melanoma (C32) cells were bought and delivered from the American Type Culture Collection (Manassas, VA, USA). HaCaT keratinocytes were ordered in AddexBio (San Diego, CA, USA). Skin cells of melanoma (A375) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco, Waltham, MA, USA) in a 5% CO_2 incubator at 37 °C. The cytotoxic activity of the essential oils was determined using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma-Aldrich) assay. Cells were seeded at a density of 1×10^4 cells per well in 96-well plates. Cells were treated with fir seed and fir cone EOs at concentrations of 0.012, 0.025, 0.05, 0.1, and 0.2 µL/mL for 24 h. EOs were dissolved in DMSO and diluted with the medium. The final DMSO concentration in the culture medium never exceeded 0.1%. A solution of 0.1% DMSO was used as the control sample. After treatment with EO, MTT solution was added, and the cells were incubated at 37 °C for 4 h in the dark. Subsequently, the medium was discarded, and violet formazan was dissolved in the solution of Sorensen's glycine buffer in DMSO. The optical density was measured in a microplate reader at a wavelength of 570 nm. The half-maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism 7 software. Independent experiments were performed in triplicate.

2.8. Antibacterial Activity

Two reference strains belonging to the strain collection of the Department of Microbiology, Immunology and Laboratory Medicine, Pomeranian Medical University, in Szczecin (Poland), were used in the current study: *Staphylococcus aureus* ATCC 29213 (methicillin-susceptible *S. aureus* (MSSA)) and *Escherichia coli* ATCC 8739. All microorganisms were cultured on Columbia agar with 5% sheep blood (bioMeriéux, Warsaw, Poland) and incubated for 18 h at 37 °C.

The minimum inhibitory concentrations (MICs) of the EOs, thymol (positive control; Sigma-Aldrich, Darmstadt, Germany), and gentamicin sulfate (positive control; solution for injections; Krka, Novo Mesto, Slovenia) against *S. aureus* and *E. coli* were evaluated via the serial microdilution method in Mueller–Hinton broth (MHB) (Sigma-Aldrich, Darmstadt, Germany). The study was performed according to the Clinical and Laboratory Standards Institute recommendations [18–20]. In brief, 50 µL of a suitable concentration of EOs/thymol/gentamicin sulfate was added to a microwell plate. Concentrations of EOs from 250 to 0.12 µL/mL were made using Tween 80 (1.0%, *v*/*v*) (Sigma-Aldrich, Darmstadt,

Germany). Concentrations of thymol from 625 to 1.22 µg/mL were made using dimethyl sulfoxide (DMSO) (2.0%, v/v) (Loba Chemie, Mumbai, India). Moreover, concentrations of gentamicin sulfate from 625 to 0.31 µg/mL were prepared. In the next step, 50 µL of microbial suspension at 10^6 CFU/mL was added to the microplate. After incubation (18 h at 37 °C), the MICs for the EOs/thymol/gentamicin sulfate were estimated via a resazurin (Sigma-Aldrich, Darmstadt, Germany) assay [21,22]. The bacteria suspension with 1.0% (v/v) Tween 80 or 2.0% (v/v) DMSO was regarded as a negative control.

Next, the minimum bactericidal concentrations (MBCs) were evaluated by transferring 20 µL from the microwells that displayed no signs of growth to a new 96-well microplate containing MHB [20–22]. The microplate was incubated for 18 h at 37 °C. The MBCs were examined in the wells in which no bacterial growth was noticed. To evaluate the effectiveness of the EOs/thymol/gentamicin sulfate, the MBC/MIC ratio was determined (MBC/MIC ratio $\leq$ 4—bactericidal effectiveness; MBC/MIC ratio > 4—bacteriostatic effectiveness) [23].

2.9. Statistical Analysis

Statistical analysis was performed using GraphPad Prism software version 7.04 (GraphPad Software, San Diego, CA, USA). A one-way ANOVA followed by Tukey's test was used. Data were reported as means ± standard deviations. Values of $p < 0.05$ were considered statistically significant.

3. Results and Discussion

3.1. Contents and Compositions of Volatile Compounds from Seeds and Cones of Greek Fir

The below-described experiments are the continuation of the research on EOs hydrodistilled from seeds and cones (cone scales) from different coniferous trees [18,24–26]. Many of these plant materials are unknown, probably due to difficulties during the collection of cones with ripe seeds.

The essential oil (EO) isolated from *Abies cephalonica* seeds displayed a high yield, nearly 30 times greater than that obtained from the cone scales (0.58–0.66%; averaging 0.62%). In fact, it reached an impressive level of 18.22%, with a range between 17.46% and 19.13%. The seed essential oil possessed an alluring, forest-fresh, citrus-like aroma, in contrast to the less appealing, resinous, earthy scent with a hint of lemon notes found in the cone scale EO. This difference in aroma is attributed to the seed oil's lower limonene content and the higher concentration of high-boiling volatile compounds, which were detected in the cone scale EO. Notably, the yield of *Abies cephalonica* seed EO (18.2%) surpassed those of previously reported *Abies concolor* seed EO (5.4%) and *Abies koreana* seed EO (4.9%) [18,24]. It also outperformed the most aromatic material to date:*Abies alba* seed EO (7.4–14.3%) [25].

Through chromatographic and spectroscopic techniques, we successfully identified a total of 101 compounds within the EOs isolated from the Greek fir. These compounds constituted 99.4% of the total seed EO composition and 98.3% of the total cone scale EO composition. Chromatograms of both EOs are shown in Figure 1. Additionally, the structures of 13 volatiles in the Greek fir seed EO were confirmed using ^{1}H-NMR spectroscopy.

The specific compounds identified in both the seed and cone scale EOs are listed in Table 1. In both the seed and cone EOs, the predominant group of terpenes detected were monoterpene hydrocarbons, constituting 95.6% of the total seed EO and 88.6% of the total cone scale EO. This composition aligns with those of previously published *Abies cephalonica* needle or bark EOs, in which the concentration of this terpene group typically fell within the range of 80–90% [1,7,12–15]. Monoterpene hydrocarbons were similarly the primary chemical group found in the EOs obtained from the seeds and cones of all the previously explored fir species: *Abies concolor* [18], *Abies koreana* [24], and *Abies alba* [25].

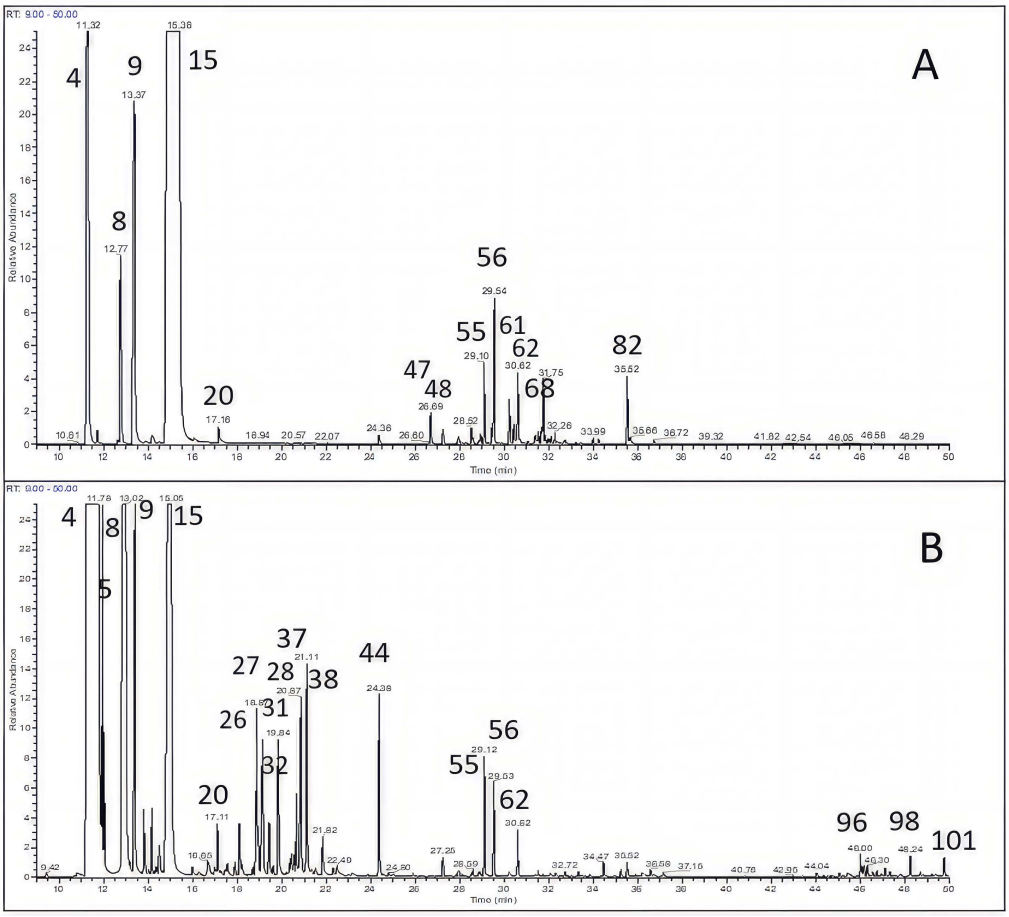


Figure 1. The gas chromatograms of (**A**) seed and (**B**) cone essential oils (numbers refer to compounds listed in Table 1).

Table 1. The chemical compositions of the Greek fir essential oils isolated from seeds and cone scales.

No.	Compound		SEED	CONE	RI $_{exp.}$	RI $_{lit.}$	Identification Method
			[%]	[%]			
1	Santene	mt	n.d.	tr.	878	884	RI, MS
2	Tricyclene	mt	tr.	0.1	919	927	RI, MS
3	α-Thujene	mt	tr.	n.d.	930	932	RI, MS
4	α-Pinene	mt	8.8	54.0	943	936	RI, MS
5	Camphene	mt	0.1	1.1	948	950	RI, MS
6	Thuja-2,4-(10)-diene	mt	tr.	0.6	950	946	RI, MS
7	Sabinene	mt	tr.	tr.	964	973	RI, MS
8	β-Pinene	mt	1.5	14.2	976	978	RI, MS
9	β-Myrcene	mt	4.0	2.6	986	987	RI, MS
10	α-Phelandrene	mt	tr.	n.d.	999	1002	RI, MS
11	δ-Car-3-ene	mt	0.2	0.3	1005	1010	RI, MS
12	α-Terpinene	mt	tr.	0.1	1010	1013	RI, MS
13	p-Cymene	mt	tr.	0.2	1013	1015	RI, MS
14	*p*-Menth-1-ene	mt	n.d.	tr.	1018	1017	RI, MS
15	Limonene	mt	80.4	14.8	1028	1025	RI, MS
16	γ-Terpinene	mt	0.2	0.1	1051	1051	RI, MS
17	Myrtenol	mto	n.d.	0.1	1059	1178	RI, MS
18	*cis*-Verbenol	mto	n.d.	0.2	1068	1132	RI, MS
19	*p*-Cymenene	mt	n.d.	0.1	1077	1075	RI, MS
20	Terpinolene	mt	0.2	0.2	1080	1081	RI, MS
21	α-Pinene oxide	mto	n.d.	0.1	1083	1082	RI, MS
22	Linalool	mto	n.d.	0.1	1091	1086	RI, MS
23	α-Fenchol	mto	n.d.	0.1	1099	1099	RI, MS
24	α-Campholenal	mto	n.d.	0.3	1105	1105	RI, MS

Table 1. *Cont.*

No.	Compound		SEED [%]	CONE [%]	RI $_{exp.}$	RI $_{lit.}$	Identification Method
25	Camphor	mto	n.d.	0.1	1120	1123	RI, MS
26 + 27	*trans*-Pinocarveol + *trans*-Verbenol	mto	n.d.	1.0	1124	1126 + 1137	RI, MS
27 + 28	*trans*-Verbenol + α-Phellandrene-8-ol	mto	n.d.	0.9	1131	1137 + 1140	RI, MS
29 + 30	Pinocamphone + Pinocarvone	mto	n.d.	0.3	1139	1140 + 1147	RI, MS
31 + 32	β-Phellandrene-8-ol + Borneol	mto	n.d.	0.8	1149	1150 + 1150	RI, MS
32	Borneol	mto	tr.		1149	1150	RI, MS
33	Terpinen-4-ol	mto	tr.	tr.	1160	1164	RI, MS
34	p-Cymen-8-ol	mto	n.d.	0.2	1165	1169	RI, MS
35	Isopinocarveol	mto	n.d.	0.1	1168	1170	RI, MS
36	Myrtenal	mto	n.d.	0.3	1170	1172	RI, MS
37	α-Terpineol	mto	tr.	1.0	1176	1176	RI, MS
38	Myrtenol	mto	n.d.	1.0	1182	1178	RI, MS
39	Verbenone	mto	n.d.	0.1	1189	1183	RI, MS
40	*cis*-Carvotanacetone	mto	n.d.	0.1	1193	1195	RI, MS
41	*trans*-Carveol	mto	n.d.	0.2	1201	1200	RI, MS
42	*cis*-Carveol	mto	n.d.	0.1	1214	1210	RI, MS
43	Carvone	mto	n.d.	0.1	1219	1214	RI, MS
44	Bornyl acetate	mto	0.1	0.7	1270	1270	RI, MS
45	*trans*-Pinocarveyl acetate	mto	n.d.	tr.	1281	1287	RI, MS
46	δ-Elemene	st	n.d.	tr.	1311	1334	RI, MS
47 + 48	δ-Elemene + α-Terpinyl acetate	st + mto	0.2	n.d.	1334	1340 + 1335	^{1}H, RI, MS
49	α-Longipinene	st	0.1	0.1	1350	1360	RI, MS
50	Cyclosativene	st	tr.	tr.	1368	1378	RI, MS
51	α-Ylangene	st	tr.	n.d.	1371	1376	RI, MS
52	Longicyclene	st	n.d.	tr.	1371	1382	RI, MS
53	β-Elemene + Sativene	st	0.1	tr.	1389	1389 + 1394	^{1}H, RI, MS
54	β-Longipinene	st	0.1	tr.	1397	1403	RI, MS
55	Longifolene	st	0.4	0.5	1404	1411	^{1}H, RI, MS
56	(*E*)-β-Caryophyllene	st	0.7	0.4	1417	1421	^{1}H, RI, MS
57	Aristolene	st	n.d.	tr.	1437	1423	RI, MS
58	γ-Elemene	st	tr.	n.d.	1428	1429	RI, MS
59	Guaia-6,9-diene	st	0.2	n.d.	1438	1443	RI, MS
60	Isogermacrene D	st	0.1	n.d.	1444	1455	RI, MS
61 + 62	Cadina-3,5-diene + α-Humulene	st	0.5	n.d.	1450	1450 + 1455	RI, MS
62	α-Humulene	st	n.d.	0.2	1450	1455	^{1}H, RI, MS
63	Alloaromadendrene	st	tr.	n.d.	1452	1462	RI, MS
64	Cadina-4,11-diene	st	0.1	n.d.	1474	1462	RI, MS
65	2-*epi*-(*E*)-β-Caryophyllene	st	0.1	n.d.	1478	1467	RI, MS
66	α-Amorphene	st	n.d.	tr.	1474	1477	RI, MS
67	γ-Muurolene	st	0.1	n.d.	1482	1474	RI, MS
68	β-Cadinene	st	0.3	n.d.	1484	1483	RI, MS
69	β-Selinene	st	0.1	n.d.	1487	1486	RI, MS
70	α-Selinene	st	tr.	n.d.	1491	1494	RI, MS
71	Dihydroagarofuran	st	0.1	n.d.	1495	1500	^{1}H, RI, MS
72	γ-Cadinene	st	0.1	n.d.	1499	1507	RI, MS
73	β-Bisabolene	st	tr.	tr.	1501	1503	RI, MS
74	7-epi-α-Selinene	st	tr.	n.d.	1513	1519	RI, MS
75	δ-Cadinene	st	tr.	n.d.	1515	1520	RI, MS
76	*trans*-α-Bisabolene	st	n.d.	tr.	1533	1530	RI, MS
77	β-Elemol	sto	tr.	n.d.	1537	1541	^{1}H, RI, MS
78	(*E*)-Nerolidol	sto	tr.	tr.	1550	1553	^{1}H, RI, MS
79	Eudesma-5,7(11)-diene	st	tr.	n.d.	1555	1543	RI, MS
80	(*E*)-β-Caryophyllene epoxide	sto	tr.	0.1	1570	1578	^{1}H, RI, MS
81	Humulene epoxide	sto	n.d.	tr.	1595	1602	RI, MS
82	Selin-6-en-4-ol	sto	0.3	0.1	1604	1612	RI, MS
83	Selin-7(11)-en-4α-ol	sto	n.d.	tr.	1609	1617	RI, MS
84	γ-Eudesmol	sto	tr.	n.d.	1616	1618	^{1}H, RI, MS
85	1-*epi*-Cubenol	sto	n.d.	tr.	1617	1623	RI, MS
86	Cubedol	sto	n.d.	tr.	1619	1630	RI, MS
87	Cubenol	sto	tr.	n.d.	1622	1630	RI, MS
88	T-Cadinol + T-Muurolol	sto	n.d.	tr.	1628	1633 + 1633	RI, MS
89	β-Eudesmol	sto	tr.	n.d.	1640	1641	RI, MS
90	α-Cadinol	sto	n.d.	tr.	1640	1643	RI, MS
91	Intermedeol	sto	tr.	tr.	1645	1657	RI, MS
92	(*Z*,*E*)-Farnesal	sto	tr.	n.d.	1680	1683	RI, MS
93	(*E*,*E*)-Farnesol	sto	tr.	n.d.	1706	1708	^{1}H, RI, MS

Table 1. *Cont.*

No.	Compound		SEED [%]	CONE [%]	RI $_{exp.}$	RI $_{lit.}$	Identification Method
94	(*E*,*E*)-Farnesal	sto	tr.	n.d.	1720	1722	^{1}H, RI, MS
95	(*E*,*E*)-Farnesyl acetate	sto	tr.	n.d.	1826	1822	^{1}H, RI, MS
96	Manoyl oxide	dto	n.d.	0.1	1988	2007	RI, MS
97	Isopimara-7,15-diene	dt	n.d.	tr.	1994	1999	RI, MS
98	Abieta-7,13-diene	dt	n.d.	0.1	2080	2084	RI, MS
99	Abieta-8(14),13(15)-diene	dt	tr.	0.1	2144	2152	RI, MS
100	Dehydroabietal	dto	n.d.	0.1	2238	2260	RI, MS
101	Abietal	dto	n.d.	0.3	2291	2261	RI, MS
Sum of identified compounds			99.4	98.3			
	Σ mt		95.4	88.4			
	Σ mto		0.1	7.3			
	Σ st		3.3	1.2			
	Σ sto		0.5	0.2			
	Σ dt		tr.	0.2			
	Σ dto		0	0.5			
	Yield of essential oil (%)		18.22	0.62			

RI $_{lit.}$—literature retention index on non-polar column; RI $_{exp.}$—experimental retention index on non-polar column; n.d.—not detected; tr.—trace (amount lower than 0.05%); MS—mass spectrometry; ^{1}H—^{1}H-NMR spectroscopy; mt—monoterpene hydrocarbons; mto—oxygenated derivatives of mt; st—sesquiterpene hydrocarbons; sto—oxygenated derivatives of st; dt—diterpene hydrocarbons; dto—oxygenated derivatives of dt.

The predominant monoterpene hydrocarbon and, at the same time, the main constituent of the tested seed essential oil was limonene (80.4%). In contrast, the EO from cone scales primarily consisted of α-pinene (54.0%), limonene (18.8%), and β-pinene (14.2%). It is worth noting that in both the tested EOs, the levorotatory forms of limonene, α-pinene, β-pinene, and camphene were the dominant enantiomers, accounting for 95.2–100.0% of the total EO composition. Detailed results are presented in Table 2.

Table 2. Contribution of the levorotatory and dextrorotatory forms of enantiomers (%) with enantiomeric excess (*ee*) of the main Greek fir seed and cone monoterpenes.

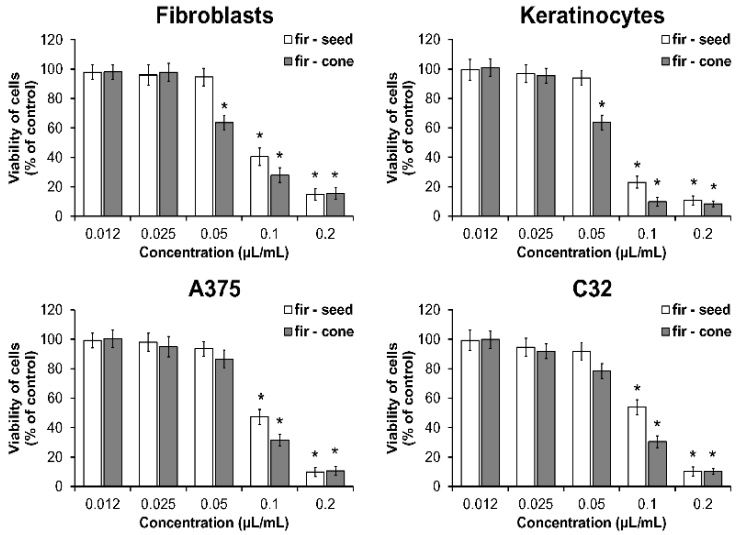
Enantiomer/Enantiomeric Excess	*Abies cephalonica*	
	Seed	Cone
(S)-(−)-Limonene (%)	97.6	93.5
(R)-(+)-Limonene (%)	2.4	6.5
ee	95.2	87.0
(1S,5S)-(−)-α-Pinene (%)	90.0	91.4
(1R,5R)-(+)-α-Pinene (%)	10.0	8.6
ee	94.2	82.8
(S)-(−)-Camphene (%)	-	81.8
(R)-(+)-Camphene (%)	-	18.2
ee	-	
(1S,5S)-(−)-β-Pinene (%)	99.8	93.3
(1R,5R)-(+)-β-Pinene (%)	0.2	6.7
ee	100.0	86.6

Limonene, particularly its levorotatory enantiomer, was a key flavor compound in both the seed and cone EOs of Greek fir, with enantiomeric excess (ee) values of 97.6% and 93.5%, respectively. This pattern of limonene dominance has also been observed in essential oils from Californian [18], silver [24,25], and Korean [24] fir seeds. Despite variations in the populations of Greek fir trees, the main constituents of monoterpene hydrocarbons, including limonene and α- and β-pinene, remained consistent in our *Abies cephalonica* cone

oils, mirroring previous findings on needle and bark essential oils from different Greek fir populations [1,7,12–15].

3.2. Cytotoxic Activity

The cytotoxic effects of the fir seed and fir cone EOs in the range of 0.012–0.2 µL/mL were analyzed using the MTT assay in skin fibroblasts, keratinocytes, and two melanoma cell lines: A375 and C32. As shown in Figure 2, both EOs induced a decrease in the cell viability in a concentration-dependent manner. However, the cone essential oil was slightly more cytotoxic than the seed EO. This is consistent with our previous studies on *Pinaceae* family members, which showed that the EO from the cones of *Abies concolor* is slightly more cytotoxic than the EO from its seeds [18]. The cone EO of fir is also almost two times more cytotoxic to fibroblasts than EOs from *Picea pungens*, *Picea orientalis*, and *Abies concolor* cones [18,26]. Neither the cone nor seed EOs had selective effects on melanoma cells when compared with normal cells. The lack of selective reduction in the cancer cell viability was shown previously in a study on EOs from *Abies alba* and *Abies koreana* seeds and cones in breast cancer [24,25].



	IC_{50} (µL/mL)	
	fir - seed	fir - cone
Fibroblasts	0.093 ± 0.009	0.067 ± 0.006
Keratinocytes	0.080 ± 0.006	0.057 ± 0.007
A375	0.095 ± 0.007	0.084 ± 0.010
C32	0.103 ± 0.101	0.076 ± 0.007

Figure 2. The viability of skin fibroblasts, keratinocytes, and melanoma cells (A375, C32) treated with fir seed and fir cone EOs for 24 h in MTT assay. The data are presented as the mean ± standard deviation of three independent experiments performed in triplicate (* $p < 0.05$ compared to the 0.1% DMSO-treated group).

3.3. Antimicrobial Activities of Greek Fir Essential Oils

Seed and cone scale EOs of *Abies cephalonica* were evaluated for their antibacterial effectiveness against two reference strains: *S. aureus* ATCC 29213 and *E. coli* ATCC 8739. In the present study, it was shown that both reference strains were susceptible to the tested EOs. The results exhibited that the cone EO had greater antibacterial activity than that isolated from seeds; however, both were characterized by bactericidal effectiveness. Moreover, in comparison to the thymol and gentamicin sulfate (positive controls), the cone EO exhibited higher and lower antibacterial activities, respectively. The detailed results of the experiments are shown in Table 3.

According to the available literature, there are few data on the antibacterial effectiveness of *Abies cephalonica* EO against microorganisms; however, in experiments that were based on EO from the needles of twigs, Mitić et al. [7] showed that the MICs of Greek fir EO against the *S. aureus* ATCC 6538 and *E. coli* ATCC 8739 strains were 0.62 and 10.0 mg/mL, respectively. Comparable results were observed in a study by Tsasi et al. [1], in which the antimicrobial activity of Greek fir needle EO was confirmed against *S. aureus* ATCC 6538 and *E. coli* ATCC 35210 (MIC = 2.5 ± 0.010 mg/mL). It is worth emphasizing that the MIC

results obtained in the current study were lower in comparison to the abovementioned data but quite similar to the results obtained in our former studies [18]. We proved the antimicrobial properties (the MIC ranged from 25 to 33 µL/mL) of *Abies concolor* EOs isolated from seeds and cones against the *E. coli* ATCC 25922 and *S. aureus* ATCC 43300 strains.

Table 3. The antibacterial effectiveness of the *Abies cephalonica* seed and cone scale essential oils (seed EO, cone EO) against reference strains.

Substance	MIC [2]	MBC [3]	MBC/MIC Ratio [4]	Effectiveness
Staphylococcus aureus ATCC 29213				
Seed EO	3.91 ± 0.00 [A]	15.63 ± 0.00 [A]	4	Bactericidal
Cone EO	0.49 ± 0.00 [A]	0.81 ± 0.28 [A]	2	Bactericidal
Thymol [1]	1.90 ± 0.00 [B]	3.80 ± 0.00 [B]	2	Bactericidal
Gentamicin sulfate [1]	0.31 ± 0.00 [B]	0.61 ± 0.00 [B]	2	Bactericidal
Escherichia coli ATCC 8739				
Seed EO	7.81 ± 0.00 [A]	31.25 ± 0.00 [A]	4	Bactericidal
Cone EO	1.63 ± 0.56 [A]	3.91 ± 0.00 [A]	2	Bactericidal
Thymol [1]	4.07 ± 1.41 [B]	8.13 ± 2.81 [B]	2	Bactericidal
Gentamicin sulfate [1]	0.51 ± 0.18 [B]	1.22 ± 0.00 [B]	2	Bactericidal

[1] Positive controls; [2] MIC—minimum inhibitory concentration; [3] MBC—minimum bactericidal concentration; [4] MBC/MIC ratio ≤ 4—bactericidal; MBC/MIC ratio > 4—bacteriostatic; [A] µL/mL; [B] µg/mL.

4. Conclusions

In summary, this is the first extensive study that focused on the chemical compositions and biological activities of seed and cone EOs from Greek fir. *Abies cephalonica* seeds are a valuable source of EO and possess an intriguing, fresh, forest-like scent, while the EO from cone scales contains relatively fewer volatile compounds. While the quantitative and qualitative makeup compositions of the tested seed and cone EOs differ, they both primarily consist of monoterpene hydrocarbons. The seed EO is predominantly characterized by its high limonene content, while the cone EO is composed of α-pinene, β-pinene, and limonene. Notably, the significant presence of the levorotatory form of these main terpenes is a distinctive feature of *Abies* species. The results indicate that the EOs derived from *Abies cephalonica* seeds and cone scales exhibit no adverse effects on human skin fibroblasts and keratinocytes when used at low concentrations and cultured for 24 h. Furthermore, the tested EOs, especially those from the cones, have promising antimicrobial properties, which could offer a compelling therapeutic option, particularly at a time when bacterial resistance to conventional antibiotics is on the rise. Surprisingly, the seeds of the Greek fir tree can serve as an excellent source of essential oil and enantiomerically pure natural limonene. In the current era of natural and organic product trends, this rich source of EO and (S)-limonene might serve as a viable alternative to synthetic fragrance compositions. However, the usage of Greek fir essential oil as a raw material in cosmetics needs to be controlled due to the high content of limonene, which is one of the volatile allergens according to European Union regulations. This research material is the first study on the chemical compositions and biological properties of essential oils from Greek fir.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app132413238/s1, details of gas chromatograms of EOs and fractions of seed EO as well NMR-spectra of seed EO fractions see the Supplementary Materials: Section S1.

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