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Chemical Profiling on Bioactive Stilbenoids in the Seeds of *Paeonia* Species Growing Wild in Greece

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Abstract: The seeds of *Paeonia clusii* Stern subsp. *clusii* and *Paeonia mascula* (L.) Mill. subsp. *mascula* growing wild in Greece, though not previously investigated, has been studied as a source of bioactive stilbenoids and other phenolics. Their methanol extracts were analyzed using ultra high-performance liquid chromatography—high-resolution mass spectrometry (UHPLC-HRMS), and among the identified metabolites (62), 19 paeoniflorin's derivatives, 17 flavonoids and 12 stilbenes were detected. Moreover, through classic phytochemical separation procedures, twelve among them were isolated and fully spectrally determined as *trans*-resveratrol, *trans*-resveratrol-4′-*O*-*β*-*D*-glucopyranoside, *cis*-resveratrol-4′-*O*-*β*-*D*-glucopyranoside, *trans*-gnetin-H, *trans*-*ε*-viniferin, luteolin, luteolin-3′-*O*-*β*-*D*-glucopyranoside, luteolin-3′,4′-di-*O*-*β*-*D*-glucopyranoside, apigenin, hispidulin, paeoniflorin and benzoyl-paeoniflorin. All seed extracts were measured for their total phenolic content (TPC), appearing as a rich source (116.04 and 103.63 mg GAE/g extract, respectively), followed by free radical (DPPH) scavenging capacity (75.24% and 91.54% inhibition at the concentration of 200 μg/mL). The evaluation of tyrosinase inhibition for both extracts (61% and 70%, respectively) confirmed the potential for their future application in skin health care, comparable with other paeonies of Chinese origin, which are well-known as skin whitening and anti-aging promoters.

Keywords: *Paeonia clusii* subsp. *clusii*; *Paeonia mascula* subsp. *mascula*; seeds; stilbenes; flavonoids; paeoniflorin; UHPLC-HRMS; TPC; DPPH; tyrosinase inhibition

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1. Introduction

Paeonia is the only genus in the Paeoniaceae family, consisting of ca. 33 species and 26 subspecies, distributed primarily in the temperate regions of Asia, South Europe, and North America [1]. Three sections are recognized within the *Paeonia* genus: (i) Moutan (shrubs in central and western China), (ii) Oneapia (herbaceous perennial species, endemic to West North America), and (iii) *Paeonia* (herbaceous perennials in eastern and central Asia, western Himalayas and the Mediterranean region) [1].

The paeonies of the Moutan section have a long history in China [2] as their roots are well-known herbal crude drugs in Traditional Chinese Medicine [3] and have been recognized for their analgesic, sedative, and anti-inflammatory properties.

Paeonia plants, over the past few decades, have become extremely popular due to their medicinal use and edible value [4]. The roots, leaves, flowers, and stems of paeonies have been studied extensively so far for their chemical composition and pharmacological activities [5]. The seeds of these plants have only very recently drawn attention, owing to their high content of α-linolenic acid in seed oil and their great potential as a new nutrient resource [4,6,7] and, since 2014, the seed oil of *P. suffruticosa* has been approved as a new resource food in China by the Competent Authority of the Ministry of Health.

The *Paeonia* genus is known as a unique source of the secondary metabolite of paeoniflorin belonging to monoterpene glycosides, while other secondary metabolites such as

flavonoids (mostly from flowers), stilbenes (with tissue distribution uniquely in seeds), triterpenoids, steroids (from roots, leaves, flowers) have also been isolated so far [4]. A large number of references in the international literature have been reported, indicating the biological activities of paeonies' different metabolites and extracts toward in vitro and in vivo test models, among which anti-inflammatory, analgesic, antioxidant, antivirus, and cytotoxic activities are listed [8,9].

Among phytochemical studies on 23 *Paeonia* plants, 23 taxa—19 species and 6 varieties—have confirmed the identification of paeoniflorin, in all examined samples, as a unique secondary metabolite in the Paeoniaceae family [10], which is also considered a chemotaxonomic marker [11]. It is noteworthy that in the Paeoniaceae family, the division in sections was based on the content of the metabolite of paeonol, which is mostly found in the woody section of Moutan, with low contents present in the herbaceous section of *Paeonia*, and absence in section Onaepia [11].

A total of eight paeony species are botanically determined in Greece, all of which belong to the herbaceous section *Paeonia*, identified as the following: *P. clusii* subsp. *clusii*, *P. clusii* subsp. *rhodia*, *P. mascula* subsp. *mascula*, *P. mascula* subsp. *hellenica*, *P. mascula* subsp. *icarica*, *P. mascula* subsp. *russi*, *P. peregrina*, and *P. parnassica* [12,13].

Paeonia clusii subsp. *clusii* is a relatively low (25–50 cm) species of peony with scented white flowers of up to 10 cm in diameter, surrounded by yellow stamens and red spots. This species is endemic to the islands, mostly of Crete and Karpathos (Greece). It has pinkish-purplish stems up to 30 cm long and glaucous-dissected leaves [12]. Only the roots of this taxon have been studied phytochemically, while in vitro antimicrobial as well as prophylactic anticonvulsant properties have been reported so far [14,15].

Paeonia mascula (L.) Mill. subsp. *mascula* is the most widespread wild paeony in Greece. It is a perennial plant (0.5–1.5 m) with purplish-red flowers (diameter of 9–12 cm) and leaves divided into three segments. It is found in central Greece, from the Ionian to the Aegean islands (Lesvos and Samos) [12]. Very recently, different extracts of the aerial parts of the plant growing in Turkey (no subspecies specified) have been analyzed using LC-DAD-ESI and LC-APCI-MS, while their antioxidant, enzyme inhibitory, and in vitro cytotoxic properties have been evaluated. Hexagalloyl-, pentagalloyl- and tetragalloyl-glucoses, together with gallic acid, paeoniflorin, and quercetin-3-*O*-glucoside, have been identified as the most abundant metabolites [16].

Until now, the qualitative screening of *Paeonia* seed extracts has been performed using UHPLC coupled with time-of-flight mass spectrometry (UPLC-QTOF-MS) [17–20] or Orbitrap analyzers [21] on different paeonies, such as *P. lactiflora, P. ostii and P. rockii* and very recently from our scientific team on *Paeonia clusii* subsp. *rhodia* [13].

This study contributes to revealing the phytochemical fingerprint of the seeds, not previously studied, of wild-growing *Paeonia clusii* subsp. *clusii* and *Paeonia mascula* (L.) Mill. subsp. *mascula* in Greece and to evaluate their composition, potential antioxidant activity, their phenolic content, and anti-tyrosinase activity.

2. Materials and Methods

2.1. Chemicals and Reagents

All the chemicals and reagents were purchased from Merck (Darmstadt, Germany) (ethanol absolute, Folin–Ciocalteu reagent, gallic acid, sodium carbonate (Na_2CO_3)), Carlo Erba Reagents (Val-de-Reuil, France) (dimethyl sulfoxide (DMSO)), Fisher Scientific (Loughborough, UK) (methanol high-performance liquid chromatography (HPLC)—grade), and Glentham Life Sciences (Corsham, UK) (2,2-diphenyl-1-picrylhydrazyl (DPPH•)).

2.2. Plant Material

Aerial parts with seeds of *Paeonia clusii* Stern subsp. *clusii* were collected from the island of Crete in May 2020 by Ms Klontza V. (MSc, Department of Pharmacy, National and Kapodistrian University of Athens, Zografou, Greece) while *Paeonia mascula* (L.) Mill. subsp. *mascula* were collected from Lesvos Island in July 2020 by Ms Ouzounelli M.

(Pharmacist). The samples were identified botanically by Dr Bazos I. (Section of Ecology & Systematics, National and Kapodistrian University of Athens, Greece). The seeds of these plants were kept in a shadowy and dry place, and they were ground by a laboratory mill (Allenwest-EAC Ltd., Brighton, UK) and stored in darkness at 25 °C until the extractions were performed.

2.3. Preparation of the Methanol Extract

Dried, grounded seeds of *P. clusii* subsp. *clusii* (9.2 g) and *P. mascula* subsp. *mascula* (4.1 g) were successively extracted (1:10) in methanol (100% MeOH) via immersion in the solvent, three times for 24 h each time, at room temperature. The filtration and in vacuo evaporation of these solvents resulted in the crude extract of MeOH (4.3 g and 1.6 g, respectively).

2.4. UHPLC-HRMS Analysis

Ultra-high-performance liquid chromatography was performed on a Vanquish UHPLC system (Thermo Fisher Scientific, Germering, Germany) Using an Accucore Vanquish UPLC C18 (2.1 × 50 mm, 1.5 µm) reverse-phased column (Thermo Fisher Scientific, Germering, Germany) and high-resolution mass spectrometry was performed on an Orbitrap Exactive Plus mass spectrometer (Thermo Scientific, Germany) as described previously [13].

2.5. Fractionation and Purification Procedures

A part of the methanolic extract of *P. clusii* subsp. *clusii* (3 g) was subjected to vacuum liquid chromatography (silica gel 60 H), eluted by organic solvents with the following increasing polarities: cyclohexane:CH_2Cl_2 (100:0 to 0:100), CH_2Cl_2: ethyl acetate (100:0 to 0:100) and ethyl acetate: MeOH (100: 0 to 70:30). This resulted in 21 combined fractions after TLC monitoring (C1-C21). Fractions C5-C6 were combined (47.2 mg) and were further purified via preparative silica gel TLC, developed in CH_2Cl_2: ethyl acetate (50:50) to afford the compounds *trans*-resveratrol (14.7 mg) and *trans*-gnetin-H (12.4 mg). Fraction C8 was a mixture of flavonoids apigenin and hispidulin (18.3 mg). Fraction C13 was identified as compound paeoniflorin (35 mg). Fraction C14 (52.2 mg) was further purified using preparative cellulose TLC and developed in H_2O:AcOH (70:30) to afford luteolin (8.2 mg), luteolin-3′-*O*-*β*-*D*-glucopyranoside (5.8 mg), and fraction C16 (43 mg) gave *trans*-resveratrol-4′-*O*-*β*-*D*-glucopyranoside (12.4 mg) and luteolin-3′,4′-di-*O*-*β*-*D*-glucopyranoside (5.4 mg), after extraction with MeOH. A part of the *P. mascula*, the subsp. *mascula* methanolic extract (0.4 g), was subjected to vacuum liquid chromatography (silica gel 60H) and eluted by the following organic solvents with increasing polarities: cyclohexane:CH_2Cl_2 (100:0 to 0:100), CH_2Cl_2: ethyl acetate (100:0 to 0:100) and ethyl acetate: MeOH (100:0 to 50:50) giving 28 fractions after TLC monitoring (M1-M28). Among them, fraction M5 was identified as *trans*-resveratrol (2.1 mg), while fraction M6 was found to be *trans*-*ε*-viniferin (2.0 mg). Fraction M10 contained luteolin (2.5 mg), and fraction M11 was identified as gnetin-H (2.7 mg). Furthermore, fraction M13 was identified as benzoyl-paeoniflorin (13.0 mg), fraction M17 as *cis*-resveratrol-4′-*O*-*β*-*D*-glucopyranoside (2.8 mg), and fraction M19 as paeoniflorin (9.1 mg).

2.6. Nuclear Magnetic Resonance (NMR)

Moreover, 1D (^{1}H-NMR, ^{13}C-NMR) and 2D-NMR spectra (COSY, HSQC, HMBC) spectra were recorded on Bruker Avance III 400 MHz and Bruker Avance II 200 MHz (Bruker BioSpin, Rheinstetten, Germany) spectrometers, using methanol-d_4 as a solvent. The chemical shift was expressed in parts per million (ppm), and all coupling constants (*J*) were expressed in Hertz.

2.7. Total Phenolic Content (TPC)

The total phenolic content of the samples was determined using the Folin–Ciocalteu method, as previously described [13]. We conducted measurements three times for each

sample, and the total phenolic content was then quantified and reported in milligrams Equivalent to Gallic Acid (GAE) per gram of the dry extract. These results are presented as means with accompanying standard deviations, with a sample size of three (n = 3).

2.8. DPPH (2,2-DiPhenyl-1-PicrylHydrazyl) Assay

We assessed the antioxidant activity of the samples using the DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay, as described in the relevant literature [13]. The samples were examined at ultimate concentrations of 200 µg/mL, 100 µg/mL, and 50 µg/mL, performing three separate analyses for each concentration. The results are reported as averages with a standard deviation derived from a sample size of three (n = 3).

2.9. Tyrosinase Inhibition

The methanol extracts of the seeds were investigated for their potential to inhibit the oxidation of L-DOPA (L-3,4-dihydroxyphenylalanine) to dopaquinone and subsequently to dopachrome via the tyrosinase enzyme as described previously [13]. Samples were assessed at a concentration of 300 µg/mL in triplicate, blank samples were included, and kojic acid was used as the positive control. The results are presented as mean values accompanying standard deviations (n = 3).

3. Results

3.1. Identification of Secondary Metabolites

Mass spectral analysis was performed to identify the bioactive compounds in the extracts of seeds for the two *Paeonia* species. Sixty-five bioactive compounds (Table 1) were tentatively identified by comparing mass spectral data with the literature as well as from databases such as the Dictionary of Natural Products [22,23], Metlin, and GNPS Public Spectral Libraries [24].

Table 1. Secondary metabolites of the methanolic extract of *P. clusii* subsp. *clusii* [a] and *P. mascula* subsp. *mascula* [b] seeds using UHPLC-HRMS in negative mode.

No	[a]	[b]	Compound	Molecular Formula	*m/z*	Ion Mode	MS/MS Fragment Mass	Literature
1	√		bis-hexoses	$C_{12}H_{22}O_{11}$	341.1092	$[M-H]^-$	89, 59	[25]
2	√	√	trisaccharides	$C_{18}H_{32}O_{16}$	533.1730	$[M + FA-H]^-$	341, 179	[25]
3	√		quinic acid	$C_7H_{12}O_6$	191.0554	$[M-H]^-$	85, 127, 173	[18]
4	√		malic acid	$C_4H_6O_5$	133.0132	$[M-H]^-$	115, 71, 133, 89	[18]
5	√		citric acid	$C_6H_8O_7$	191.0192	$[M-H]^-$	85, 127, 111, 173, 150	[18]
6	√		desbenzoylpaeoniflorin	$C_{16}H_{24}O_{10}$	375.1301	$[M-H]^-$	165, 345, 89	[18,26]
7	√	√	glucogallin	$C_{13}H_{16}O_{10}$	331.0676	$[M-H]^-$	169, 211, 151, 271	[18,26]
8	√		gallic acid	$C_7H_6O_5$	169.0135	$[M-H]^-$	169	[18,26]
9	√	√	benzoylpaeoniflorin	$C_{30}H_{32}O_{12}$	583.1892	$[M-H]^-$	165, 121, 333	[18]
10	√		deoxyloganin acid	$C_{16}H_{24}O_9$	405.1408	$[M + FA-H]^-$	179, 197	[25]
11	√	√	loganin	$C_{17}H_{26}O_{10}$	389.1455	$[M-H]^-$	69, 163, 309, 181	[26]
12	√		oxypaeoniflorin isomer	$C_{23}H_{28}O_{12}$	495.1512	$[M-H]^-$	137, 281, 165	[21,27]
13	√		methyl gallate	$C_8H_8O_5$	183.029	$[M-H]^-$	183, 168	[18,28]

Table 1. *Cont.*

No	[a]	[b]	Compound	Molecular Formula	*m/z*	Ion Mode	MS/MS Fragment Mass	Literature
14	√		oxypaeoniflorin isomer	$C_{23}H_{28}O_{12}$	495.1510	$[M-H]^-$	137, 281, 165	[21,27]
15	√	√	resveratrol diglucoside	$C_{26}H_{32}O_{13}$	597.1836	$[M + FA-H]^-$	227	[25]
16	√		mudanpioside E	$C_{24}H_{30}O_{13}$	525.1611	$[M-H]^-$	121, 167	[18]
17	√	√	isomaltopaeoniflorin	$C_{29}H_{38}O_{16}$	687.2152	$[M + FA-H]^-$	121, 165	[18,29,30]
18	√	√	paeoniflorin isomer	$C_{23}H_{28}O_{11}$	479.1559 525.1617	$[M-H]^-$, $[M + FA-H]^-$	121, 165	[18,27]
19	√	√	trigalloyl glucose	$C_8H_8O_2$	635.0906	$[M-H]^-$	169, 313, 465	[2,31,32]
20	√	√	glucopyranosylalbiflorin	$C_{29}H_{38}O_{16}$	687.2152	$[M + FA-H]^-$	121, 165	[18,29,30]
21	√	√	*cis*-resveratrol-4′-*O*-*β*-*D*-glucopyranoside	$C_{20}H_{22}O_8$	389.1248	$[M-H]^-$	227, 185	[18,32]
22	√	√	methyldesbenzoylpaeoniflorin	$C_{17}H_{26}O_{10}$	435.1299	$[M-H]^-$	227, 389	[18]
23	√	√	paeoniflorin/albiflorin	$C_{23}H_{28}O_{11}$	479.1559525.1618	$[M-H]^-$ $[M + FA-H]^-$	121, 165	[18]
24	√		oxypaeoniflorin	$C_{23}H_{28}O_{12}$	495.1513	$[M-H]^-$	137, 165	[13,27]
25	√		kaempferol dihexoside	$C_{30}H_{25}O_{14}$	609.1472	$[M-H]^-$	285, 447	[18,33]
26	√	√	tetragalloyl glucose	$C_8H_8O_2$	787.1014	$[M-H]^-$	169, 465, 313	[27,29,31]
27	√	√	luteolin-7-O-glucopyranoside	$C_{21}H_{20}O_{11}$	447.0936	$[M-H]^-$	285	[13,18,23]
28	√		luteolin 3′,4′-di-*O*-*β*-*D*-glucopyranoside	$C_{27}H_{30}O_{16}$	609.1462655.1524	$[M-H]^-[M + FA-H]^-$	285, 447	[18]
29	√	√	astragalin	$C_{21}H_{20}O_{11}$	447.0933	$[M-H]^-$	285	[13,18,23]
30	√	√	apigenin hexoside	$C_{21}H_{20}O_{10}$	431.0986	$[M-H]^-$	269, 121, 225	[13,33]
31	√		galloyl paeoniflorin	$C_{30}H_{32}O_{11}$	631.1676	$[M-H]^-$	169, 313, 211, 271, 121, 399	[27,29,30]
32	√	√	*trans*-resveratrol-4′-*O*-*β*-*D*-glucopyranoside	$C_{20}H_{22}O_8$	389.1249	$[M-H]^-$	227	[13,32]
33	√	√	apigenin hexoside	$C_{21}H_{20}O_{10}$	431.0987	$[M-H]^-$	269, 121, 225	[13,33]
34	√	√	kaempferol hexoside	$C_{21}H_{20}O_{11}$	447.0938	$[M-H]^-$	285	[13,33]
35	√	√	isorhamnetin glucopyranoside	$C_{22}H_{22}O_{12}$	477.1046	$[M-H]^-$	121, 315, 299	[13,34]
36	√	√	trihydroxy methoxyflavone hexoside	$C_{22}H_{22}O_{11}$	461.1096	$[M-H]^-$	283, 446	[23,29]
37	√		galloyl albiflorin	$C_{30}H_{32}O_{11}$	631.1676	$[M-H]^-$	629	[27,29,30]
38	√	√	*cis*-resveratrol	$C_{14}H_{12}O_3$	227.0712	$[M-H]^-$	185, 143, 183	[18,32]
39	√	√	luteolin-3′-*O*-*β*-*D*-glucopyranoside	$C_{21}H_{20}O_{11}$	447.0938	$[M-H]^-$	285	[18]
40	√	√	albiflorin isomer	$C_{23}H_{28}O_{11}$	525.1617	$[M + FA-H]^-$	121, 165	[18,20]

Table 1. *Cont.*

No	[a]	[b]	Compound	Molecular Formula	*m/z*	Ion Mode	MS/MS Fragment Mass	Literature
41	√		phloridzin	$C_{21}H_{24}O_{10}$	435.1297	$[M-H]^-$	167, 273	[18]
42	√		paeoniflorin isomer	$C_{23}H_{28}O_{11}$	539.1777	$[M + Hac-H]^-$	121	[18,25]
43	√	√	lactiflorin	$C_{23}H_{26}O_{10}$	507.1510	$[M + FA-H]^-$	121, 299, 284, 177	[18]
44	√		benzoyloxypaeoniflorin	$C_{30}H_{32}O_{13}$	599.1779	$[M-H]^-$	137, 121, 281	[27,29]
45	√	√	kaempferol	$C_{15}H_{10}O_6$	285.0406	$[M-H]^-$	151, 175	[18]
46	√		*cis*-ε-viniferin hexoside	$C_{34}H_{32}O_{11}$	615.1873	$[M-H]^-$	453, 347, 359, 333, 227	[18]
47	√	√	kaempferol arabinoside	$C_{20}H_{18}O_{10}$	417.0833	$[M-H]^-$	285	[29]
48	√	√	luteolin	$C_{15}H_{10}O_6$	285.0408	$[M-H]^-$	151, 175	[18]
49	√	√	methyl paeoniflorin	$C_{24}H_{29}O_{11}$	493.1722	$[M-H]^-$	121, 165	[30]
50	√	√	*trans*-resveratrol	$C_{14}H_{12}O_3$	227.0711	$[M-H]^-$	185, 143, 183	[18,32]
51	√		*trans*-ε-viniferin hexoside	$C_{34}H_{32}O_{11}$	615.1873	$[M-H]^-$	453, 347, 359, 333, 227	[18]
52	√	√	kaempferol arabinoside	$C_{20}H_{18}O_{10}$	417.0833	$[M-H]^-$	285	[29]
53	√		apigenin	$C_{15}H_{10}O_5$	269.0459	$[M-H]^-$	151, 225	[18]
54	√	√	*cis*-ε-viniferin	$C_{28}H_{22}O_6$	453.1349499.1405	$[M-H]^-[M + FA-H]^-$	347, 225, 93, 411	[35]
55	√	√	carasiphenol A	$C_{27}H_{24}O_5$	427.1555	$[M-H]^-$	119,265, 307, 145, 161, 369	[18,36]
56	√	√	mudanpioside J	$C_{31}H_{34}O_{14}$	629.1884	$[M-H]^-$	121, 165	[30]
57	√		hispidulin	$C_{16}H_{12}O_6$	299.0563	$[M-H]^-$	284	[37]
58	√		isorhamnetin	$C_{16}H_{12}O_7$	315.0515	$[M-H]^-$	300, 315, 91, 149	[13,19]
59	√	√	*trans*-ε-viniferin	$C_{28}H_{22}O_6$	453.1347	$[M-H]^-$	347, 225, 93, 411	[35]
60	√		*cis*-gnetin H	$C_{42}H_{32}O_9$	679.1984	$[M-H]^-$	93, 491, 478, 449, 357, 225, 585	[9,32]
61	√	√	*trans*-gnetin H	$C_{42}H_{32}O_9$	679.1981	$[M-H]^-$	93, 345, 225, 491, 449, 357, 585	[9,32]
62	√	√	ursolic acid	$C_{30}H_{48}O_3$	455.3537	$[M-H]^-$	455	[18]

√ = compound is present in the extract.

3.2. Isolation of Secondary Metabolites

From the *P. clusii* subsp. *clusii* methanolic extract, nine compounds belonging to three different chemical groups were isolated and fully elucidated through spectral data. Three stilbene derivatives, *trans*-resveratrol, *trans*-resveratrol-4′-*O*-*β*-*D*-glucopyranoside, gnetin-H, five flavonoids, luteolin, luteolin-3′-*O*-*β*-*D*-glucopyranoside, luteolin-3′,4′-di-*O*-*β*-*D*-glucopyranoside, apigenin, hispidulin, and the monoterpenic glucoside of paeoniflorin

were used. Moreover, from the same extract of *P. mascula* subsp. *mascula*, seven known metabolites were characterized as follows: *trans*-resveratrol, *trans*-ε-viniferin, luteolin, gnetin-H, benzoyl-paeoniflorin, *cis*-resveratrol-4′-*O*-*β*-*D*-glucopyranoside, and paeoniflorin. All structures were known and determined by means of ^{1}H- and ^{13}C-NMR with a comparison between spectroscopic data and those reported in the literature [13,38].

3.3. Total Phenolic Content (TPC) and DPPH Assay

The total phenolic content was measured in the methanol extract of seeds from the studied subspecies. It was found that both extracts contained a high phenolic content (116 and 103 mg GAE/g extract), as shown in Table 2, assuming that these subspecies are a rich source of phenolics, which is in accordance with the presented phytochemical profile.

Table 2. Determination of TPC, DPPH and tyrosinase inhibition of seeds' methanol extract of *P.clusii* subsp. *clusii* and *P. mascula* subsp. *mascula.*

Studied Extracts	TPC (mg GAE/g Extract)	% DPPH• Inhibition			% Tyrosinase Inhibition
		200 μg/mL	100 μg/mL	50 μg/mL	300 μg/mL
P. clusii subsp. *clusii*	116.04 ± 2.6	75.24 ± 0.7	43.47 ± 1.04	21.08 ± 0.16	61.39 ± 1.57
P. mascula subsp. *mascula*	103.63 ± 2.6	91.54 ± 0.94	56.04 ± 1.12	30.08 ± 1.15	70.79 ± 0.74

Results were expressed as the mean ± standard deviation (n = 3).

The antioxidative capacity of the seeds' methanol extracts was determined using the DPPH assay, and it was determined that they exhibited high free radical scavenging activity (Table 2) and, therefore, a significant antioxidative profile for both herein studied subspecies.

3.4. Tyrosinase Inhibition Activity

The tyrosinase inhibition activity of the studied methanolic extracts of *P. clusii* subsp. clusii and *P. mascula* subsp. mascula seeds are presented in Table 2.

4. Discussion

Following the UHPLC-HRMS method of analysis for methanol extracts from the seeds of *P. clusii* subsp. *clusii* and *P. mascula* subsp. *mascula* seeds, sixty-two (62) metabolites were identified, comprising mostly monoterpene glycosides, oligostilbenes, and flavonoids. In the present study, and nineteen (19) monoterpene glycosides, which were characterized to include oxypaeoniflorin, paeoniflorin, methylpaeoniflorin, benzoylpaeoniflorin, galloyl-paeoniflorin, desbenzoylpaeoniflorin, methyl-desbenzoylpaeoniflorin, albiflorin, galloyl-albiflorin, mudanpioside E, mudanpioside J, benzoyloxypaeoniflorin and their hexose derivatives. Most of them contained the common pinane skeleton of paeoniflorin, which is a major active component in all paeonies, as already stated, and is considered a chemotaxonomic marker. Paeoniflorin itself has been previously reported in the roots of *P. clusii* subsp. *clusii* [15], while all its derivatives have been identified in seed kernels of *P. lactiflora* Pall. [18]. In herbaceous peonies, benzoylpaeoniflorin, desbenzoylpaeoniflorin, albiflorin, paeoniflorin, oxypaeoniflorin, and their derivatives were isolated from the roots of *P. lactiflora* [4]. Several monoterpenes reported in this study have also been previously identified in tree peonies as mudanpiosides E & J and galloylpaeoniflorin from the roots and root cortex of *P. ostii* and *P. suffruticosa*, respectively [5,39,40]. Benzoylpaeoniflorin has been identified previously in all plant organs of *P. suffruticosa* [41].

Stilbenes isolated from the seeds of studied paeony species were recognized as resveratrol oligomers. Twelve (12) oligostilbenes were identified as *trans*- and *cis*-resveratrol as well as in the form of their dimers viniferin and carashipenol A, and trimers gnetin-H

isomers, along with their glucosides. Most of them have been previously reported in the seeds of *P. suffruticosa* and *P. lactiflora* [13].

In our study, among the identified phenolic metabolites, five (5) were detected as gallic derivatives (gallic acid, methyl gallate, glucogallin, trigalloyl glucose, and tetragalloylglucose), which is in accordance with the results of previous studies on other paeonies as well as in seed kernels and hulls of *P. lactiflora* Pall. [18].

Furthermore, seventeen (17) flavonoids, including kaempferol, luteolin, apigenin, isorhamnetin and their glucosides, methoxy-kaempferol, astragalin, hispidulin, chrysoeriol glucoside, and one trihydroxy methoxy-flavone hexoside were determined in the seeds of studied Greek paeonies. MS data were consistent with the presence of flavonoids, which were also found in the seed kernels and hulls of *P. lactiflora* Pall. [18]. Remarkably, luteolin-glucopyranosides (luteolin-3′-*O*-*β*-*D*-glucopyranoside and luteolin-3′,4′-di-*O*-*β*-*D*-glucopyranoside) have only been isolated and determined before by our group from the seeds of the Greek *Paeonia clusii* subsp. *rhodia* [13] for the first time in the Paeoniaceae family. The flavonoids apigenin and hispidulin were isolated and structurally determined in *P. clusii* subsp. *clusii* seeds in this study, while according to the literature, apigenin (4′,5,7,-trihydroxyflavone) was identified previously in flowers of *P. ostii* and is a well-known flavonoid for its interesting pharmacological activity as an antioxidant, anti-inflammatory and antihyperglycemic agent [42]. Hispidulin (6-methoxy derivative of apigenin), not previously referred to in any paeony species, is known as an effective cytotoxic agent [43].

Four more categories of secondary metabolites were also tentatively identified: two iridoid glucosides (loganin, deoxyloganin acid), one triterpene (ursolic acid), one polyphenol (phloridzin), and three carboxylic acids (quinic, malic and citric acid), all of which have been previously identified in the seed kernels of *P. lactiflora* Pall. [18].

To the best of our knowledge, there are reports that have investigated the phenolic content in peony seeds [19,44–47], most of them regarding tree peonies (section. Moutan) and only one, by our team, conducted on Greek *Paeonia* species (section *Paeonia*) [13].

In this study, the total phenolic content was measured in the methanol extracts of seeds and it was found that both investigated seeds contained a high phenolic content (116.04 ± 2.6 mg GAE/g extract and 103.63 ± 2.6 mg GAE/g extract). These results are in accordance with the above-studied phytochemical profiles, as both extracts exhibited a rich phenolic content through UHPLC-HRMS analyses (with several flavonoids and stilbenoids: luteolin, kaempferol, apigenin, hispidulin, resveratrol, viniferin, and gnetin H, etc.). Furthermore, comparing our results with bibliographic data, it was observed that the studied seeds showed a TPC value (50.45 and 40.63 mg GAE/g DW) within the range compared to those of seeds coats from tree *Paeonia* species (section Moutan), which ranged from 28.16 to 89.50 mg GAE/g DW as well as with those of the seed coats of herbaceous plants belonging to section *Paeonia* (54.28–159.36 mg GAE/g DW) [45]. They have a lower TPC content in comparison with the seeds of recently studied Greek *P clusii* subsp. *rhodia* (phenolic contents: 204.62 ± 4.0 mg GAE/g extract) [13]. The methanol extracts studied from the aerial parts of *P mascula* (not subspecies specified) of Turkish origin appeared less rich in secondary metabolites but comparable with TPC (122.45 ± 0.48 mg GAE/g extract) [16] in the presently studied seeds.

There is a large demand today to search for new bio-antioxidants from natural sources, and especially from plants, due to the toxic effects of synthetic antioxidants, which have been commonly used in the food and cosmetic industries [48]. The antioxidative capacity of seeds from Greek *Paeonia* methanol extracts was determined using the DPPH• assay, exhibiting high free radical scavenging activity, probably due to its high TPC. Among them, luteolin and other flavonoid derivatives, such as apigenin and kaempferol glucoside and diglucosides, possessed significant DPPH• radical scavenging activity in a study conducted by Zhang et al. [49], suggesting that high flavonoid content is directly related to strong antioxidant properties. Furthermore, it has been previously documented that polyphenols, such as gallic acid and methyl gallate, possess a greater capacity for scavenging free radicals compared to the well-known antioxidant compound α-tocopherol [50]. Moreover, among

stilbenes, resveratrol dimers and trimers demonstrated the strongest antioxidant activity, while resveratrol exhibited moderate activity surpassing that of its glycosides: *trans* and *cis*-resveratroloside. This suggests that the glucosylation of resveratrol reduces its antioxidant activity due to its depleted affinity for biological lipid membranes [51,52]. It is noteworthy that paeoniflorin, as the most characteristic metabolite of the genus, has been observed not to inhibit free radical DPPH [50]. Consequently, future research should delve deeper into the synergistic, antagonistic, and antioxidant activities of phenolic substances in peonies.

There are no previous studies on the antioxidant activity of *P. clusii* subp. *clusii* and *P. mascula* subsp. *Mascula,* but comparing our results with those of other paeonies, it can be concluded that they are significantly higher. Specifically, Sevim et al. [47] reported that extracts of seeds from seven herbaceous peony species (section *Paeonia*) in Turkey have demonstrated low to moderate activity in scavenging radicals—below 50% at 2000 μg/mL. In another study, Yang et al. [44] determined that the antioxidative potential of seed coats from the subgenus *Paeonia* (66.56 mg/g to 82.85 mg/g) was generally higher compared to subgenus Moutan (27.81 mg/g to 67.58 mg/g).

The comparison with closely related *P clusii* subsp. *rhodia* (19.9 ± 1.8 at 100 μg/mL) [13] showed that the herein-studied seeds exhibited higher antioxidant capacity (43% and 56% inhibition at 100 μg/mL). It is obvious that these two studied seeds from Greek paeony species may serve as good sources for extracts with high antioxidant activity.

According to the anti-tyrosinase test results, both screened methanol extracts of *P. clusii* subsp. *clusii* and *P. mascula* subsp. *mascula* showed strong inhibitory activity (61% and 70%, respectively). These results are comparable with previous ones from *Paeonia clusii* subsp. *rhodia* seeds [13], which also exerted a very strong inhibition alongside the methanolic extract of *P. mascula* from Turkey, which exerted high anti-tyrosinase activity (56.02 ± 0.51), attributed by the authors to its hexagalloyl glucose content [16].

Bioactivity assays both online and offline revealed that extracts from *P. lactiflora* display remarkable antioxidant properties, a potent antiglycation capacity, and significant inhibition of tyrosinase, cyclooxygenase-2, and collagenase enzymes. The main contributors to these antioxidants and antityrosinase effects were gallic acid derivatives, which also exhibit the potential inhibition of cyclooxygenase-2 and collagenase, although their antiglycation capacity was relatively weak. The antiglycation effects may be due to the synergistic action of gallic acid and specific flavonoids. These findings show a method for the development of natural *skin* health products using these promising sources of bioactive compounds [53].

Moreover, of the wide uses of *Paeonia*'s species as important ornamental and medicinal crops, very recent results on the seeds of Chinese paeonies of the Moutan section propose paeonies' seeds as strong skin whitening agents through both oral and external use support the health and anti-aging of skin [54,55]. The two studied species contain the luteolin-glucopyranosides (luteolin-3′-*O*-*β*-*D*-glucopyranoside and luteolin-3′,4′-di-*O*-*β*-*D*-glucopyranoside) which are mentioned in the literature for the second time in *Paeonia* sp (isolated and determined previously by our group, from seeds of the Greek *Paeonia clusii* subsp. *rhodia* [13]). Luteolin is a known metabolite for its antioxidant, anti-inflammatory, neuroprotective, antiviral, and antidiabetic activity, and further studies on the activities of its glucopyranoside derivatives should be further studied.

5. Conclusions

Paeonies' seeds have recently gained high interest from the scientific community for further exploration as a source of phenolic metabolites such as flavonoids and mostly bioactive stilbenes (resveratrol derivatives). In the framework of our continuous studies on seeds of paeonies (section *Paeonia*) growing wild in Greece, this study sheds light on the chemical composition and biological benefits of *P. clusii* subsp. *clusii* and *P. mascula* subsp. *mascula*. Sixty-two metabolites were identified and characterized by analyzing UHPLC-HRMS data from the methanol extracts of their seeds, comprising mostly paeoniflorin derivatives, oligostilbenes (resveratrol derivatives), and flavonoids (flavones and flavonols derivatives). Based on the TPC, DPPH, and tyrosinase inhibition assays, this study potentially lays a

foundation for the utilization of such seeds as a rich source of bioactive natural compounds. As skin health is a major need and concern across the world, such metabolites could be potentially applied, after further bioassays, in the field of skin care products, mostly as potential skin whitening and anti-aging candidates. Furthermore, the pleasant taste and aroma of these seeds, as well as their traditional use since antiquity as edible crude material, could further suggest their potential safe use in food supplements and/or toward other health-promoting applications.

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