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Extracts of Different Polarity of *Daphne laureola* L. as Valuable Source of Antioxidant and Neuroprotective Compounds †

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Abstract: In this study, *Daphne laureola* L., a European–Mediterranean species, was investigated for its antioxidant properties using different in vitro bioassays, namely 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Ferric Reducing Activity Power (FRAP), and β-carotene bleaching assays. The acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity using Ellman's method was also examined. The aerial parts of *D. laureola* were subjected to exhaustive and subsequent macerations with solvents at different polarities, such as methanol, dichloromethane, and *n*-hexane. Dichloromethane extract was the most promising in DPPH and FRAP tests with IC_{50} values of 32.2 µg/mL and 71.5 µM Fe(II)/g, respectively. Methanol extract showed the most promising inhibition of lipid peroxidation evaluated by β-carotene bleaching test with an IC_{50} value of 6.6 µg/mL after 30 min of incubation. This extract was the most active against AChE (IC_{50} value of 56.9 µg/mL). An interesting result was obtained against BChE by the *n*-hexane extract, with an IC_{50} value of 49.7 µg/mL. In conclusion, the results suggest that *D. laureola* may provide a substantial source of phytochemicals, which act as natural antioxidants and acetylcholinesterase inhibitors, and may be beneficial in the treatment of Alzheimer disease.

Keywords: *Daphne laureola*; antioxidant; natural compounds; Alzheimer's disease; acetylcholinesterase; butyrylcholinesterase

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1. Introduction

Daphne L. is one of the most diverse genera in the Thymelaeaceae family, with over 90 described species distributed in Asia, parts of North Africa, and Europe. Several species of this genus are used in traditional medicine for their antimicrobial, anticancer, anti-inflammatory, antitussive, and anti-rheumatic properties [1]. Terpenoids, phenols coumarins, lignans, and flavonoids are the most important classes of compounds identified in the genus *Daphne* [1].

Daphne laureola L., a European–Mediterranean species [2], is an evergreen shrub growing to 1 m with large, glabrous, and glossy leaves. Few studies are present in the literature on this Daphne species. One of these works reported interesting antioxidant and antimicrobial effects of a population of *D. laureola* from Serbia in relation to the presence of phenols and flavonoids as the most abundant constituents [3].

Alzheimer's Disease (AD) is the most common cause of dementia in the ageing population. The primarily degenerative condition is characterized by the formation of amyloid plaques, neurofibrillary tangles and loss of neurons and synapses as well. Research revealed early degeneration of cholinergic nuclei localised in the basal forebrain. Impairment of this cholinergic system is followed by disturbance of attentional processes and cognitive

decline. Acetylcholinetserase (AChE) inhibitors are currently the best-established treatment for this disease.

Despite the extensive research, only a few drugs are currently available to treat AD. In the last years, several natural compounds exhibited beneficial effects for the treatment of AD, targeting different mechanisms of action.

A wide range of natural molecules have proven to be efficient in different preclinical and clinical studies and may play an important role in the prevention and/or treatment of AD [4].

The present study provides information on antioxidant and neuroprotective properties of *D. laureola* aerial parts collected in Southern Italy. The assays applied are comprised of methods testing radicals scavenging and lipid peroxidation preventing capacities of n-hexane, dichloromethane, and methanol extracts of this plant, together with their chemical profile investigated by gas chromatographic analyses. The ability of *D. laureola* extracts to inhibit cholinesterases is also assessed.

2. Experiments

2.1. Chemicals and Reagents

Solvents of analytical grade were obtained from VWR International s.r.l. (Milan, Italy). Sodium phosphate buffer, acetylthiocholine iodide (ATCI), 5,5′-dithiobis(2-nitrobenzoic-acid) (DTNB), butyrylthiocholine iodide (BTCI), Folin–Ciocalteu reagent, $AlCl_3$, physostigmine, acetylcholinesterase (AChE) from *Electrophorus electricus* (EC 3.1.1.7, Type VI-S), butyrylcholinesterase (BChE) from equine serum (EC 3.1.1.8), butylated hydroxytoluene (BHT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene, chlorogenic acid, and quercetin were purchased from Sigma-Aldrich S.p.a. (Milan, Italy).

2.2. Plant Materials and Extraction Procedure

Daphne laureola aerial parts were collected in Sila, Calabria, Southern Italy (voucher in PI) by L. Peruzzi. Dried samples were subjected to exhaustive macerations (800 mL, 3 × 48 h) with solvents at different polarities, such as *n*-hexane, dichloromethane, and methanol. The resultant solutions were evaporated to dryness to give extracts of 2.0, 1.1, and 14.9 g for *n*-hexane, dichloromethane, and methanol extract, respectively, and extraction yields of 0.9%, 0.5%, and 6.9% for *n*-hexane, dichloromethane, and methanol extract, respectively.

2.3. Chemical Analysis

n-Hexane and dichloromethane extracts were investigated by gas chromatography-mass spectrometry (GC-MS) as previously reported [5]. Briefly, GC-MS analyses were performed on a Hewlett-Packard 6890 gas chromatograph fitted with a fused silica HP-5 capillary column (30 m length, 0.25 mm i.d., 0.25 μm film thickness). Ionization energy voltage 70 eV was used. Helium was used as carrier gas. The column temperature was initially kept at 50 °C for 5 min, and then increased to 280 °C at 13 °C/min, held for 10 min at 280 °C. Constituents were tentatively identified through gas chromatography, comparing their retention times either with those in the literature or with those of authentic compounds available in our laboratory. Further identification was made by comparing their mass spectra with either those stored in Wiley 275 library or with mass spectra from the literature and from our in-house library [6,7].

The methanol extract was analysed for its total phenols content (TPC) as previously reported [8]. The extract was tested at a concentration of 1.5 mg/mL. The absorbance was measured at 765 nm using a UV-Vis Jenway 6003 spectrophotometer (Carlo Erba, Milan, Italy). TPC was expressed as mg of chlorogenic acid equivalents (CAE)/g of dried weight (DW). The total flavonoid content (TFC) was determined spectrophotometrically by using a method based on the formation of a flavonoid–aluminium complex [8]. The absorbance was measured at 510 nm and TFC was expressed as mg quercetin equivalents (QE)/g DW.

2.4. Antioxidant Activity

The antioxidant properties of *Daphne laureola* extracts were assessed by using Ferric Reducing Antioxidant Power (FRAP), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and β-carotene bleaching tests. The FRAP reagent is a mixture of 10 mM tripyridyltriazine solution, 40 mM HCl, 20 mM $FeCl_3$, and 0.3 M acetate buffer (pH 3.6) [9]. *D. laureola* extracts at a concentration of 2.5 mg/mL in ethanol were mixed with FRAP reagent and water.

The absorption of the reaction mixture was measured at 595 nm after 30 min of incubation at 25 °C. Ethanol solutions of known Fe (II) concentration, in the range of 50–500 μM ($FeSO_4$), were used for obtaining the calibration curve. ABTS assay was applied using the methodology described by Brindisi et al. [9]. A solution of ABTS radical cation was prepared. After 12 h, the solution was diluted with ethanol to an absorbance of 0.70 at 734 nm using a UV-Vis Jenway 6003 spectrophotometer (Carlo Erba, Milan, Italy). Dilution of extracts in ethanol was added to 2 mL of diluted ABTS solution in order to test concentrations from 400 to 1 μg/mL. After 6 min, the absorbance was read at 734 nm.

The DPPH radicals scavenging activity was determined as previously reported [9]. An aliquot of 1.5 mL of 0.25 mM DPPH radical in ethanol was mixed with 12 μL of extracts in order to test concentrations in the range 1–1000 μg/mL. The absorbance was read at 517 nm.

In the β-carotene bleaching test, a mixture of linoleic acid, Tween 20, and β-carotene was prepared as previously described [9]. β-Carotene was added to linoleic acid 100% Tween 20. After evaporation of the solvent and dilution with water, the emulsion was added to a 96-well microplate containing samples in ethanol concentrations ranging from 100 to 2.5 μg/mL. The plate was incubated at 45 °C for 30 and 60 min. The absorbance was measured at 470 nm.

2.5. Acetylcholinesterase (AChE) and Butyrylcholinesterase (BChE) Inhibitory Activity

The inhibition of AChE and BChE enzymes was measured by using a modified colorimetric Ellman's method as previously reported [10]. AChE from *Electrophorus electricus* (EC 3.1.1.7, Type VI-S) and BChE equine serum (EC 3.1.1.8) were used. Acetylthiocholine iodide and butyrylthiocholine iodide were employed as reaction substrates. In brief, enzyme, samples, and phosphate buffer were mixed in a microplate and incubated in an ice bath at 4 °C. After 30 min, physostigmine was added. The reaction started by adding the substrate and the 5,5′-dithiobis(2-nitrobenzoic-acid) solution. The microplate was placed in a thermostatic water bath (Branson model 3800-CPXH, Milan, Italy) at 37 °C. After 20 min, the reaction was stopped by placing the microplate in an ice bath and by adding physostigmine. The absorbance was measured at 405 nm.

2.6. Statistical Analysis

Data are expressed as means ± standard deviation. Prism GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA, USA) was used to calculate IC_{50} values (the concentration that yielded 50% inhibition). Results were analyzed statistically using a one-way analysis of variance test (ANOVA) followed by a multicomparison Dunnett's test ($\alpha = 0.05$).

3. Results

3.1. Phytochemicals Content

n-Hexane and dichloromethane extracts of *Daphne laureola* were analysed by GC and GC-MS. Monoterpenes, sesquiterpenes, fatty acids, and sterols were identified in both extracts. In particular, the *n*-hexane extract was characterized by the presence as main constituents of different terpenes, such as germacrene D, neophytadiene, *trans*-caryophyllene, α-humulene, γ-cadinene, δ-cadinene, some alkanes (eicosane, heneicosane, pentacosane, nonacosane) and several fatty acids with their methyl esters such as myristic acid, linoleic acid, methyl palmitate, methyl linoleate, methyl linolenate, methyl stearate.

These fatty acids, together with phytol, and several sterols and triterpenes, namely stigmasterol, stigmasta-5,23-dien-3-ol, β-sitosterol, α-amyrin, and β-amyrin, characterized dichloromethane extract. The methanol extract showed a TPC of 11.5 mg chlorogenic acid equivalents (CAE)/g of dried weight (DW) and a TFC of 2.3 mg quercetin equivalents (QE)/g DW.

3.2. Antioxidant Properties

Daphne laureola extracts were investigated for their antioxidant potential by a multi-target approach, which involved the application of four in vitro assays such as ABTS, DPPH, FRAP, and β-carotene bleaching tests. *D. laureola* extracts exhibited antioxidant effects in a concentration-dependent manner. As reported in Table 1, dichloromethane extract was the most promising as radicals scavenging agent (IC_{50} values of 18.3 and 32.2 μg/mL in ABTS and DPPH tests, respectively).

Table 1. Radical scavenging activity and antioxidant properties of *Daphne laureola* extracts.

D. laureola	DPPH Assay (IC_{50} μg/mL)	ABTS Assay (IC_{50} μg/mL)	FRAP Test [1] (μM Fe(II)/g)	β-Carotene Bleaching Test (IC_{50} μg/mL)	
				30 min	60 min
n-Hexane	74.3 ± 1.8 ****	47.4 ± 1.2 ****	2.2 ± 0.5 ****	42.9 ± 1.0 ****	46.3 ± 1.4 ****
Dichloromethane	32.2 ± 0.9 ****	18.3 ± 0.7 ****	71.5 ± 2.1	8.5 ± 0.8 ***	9.4 ± 0.4 ****
Metanolo	63.6 ± 1.2 ****	22.5 ± 1.1 ****	4.1 ± 0.8 ****	6.6 ± 0.6 ***	9.9 ± 0.7 ****
Positive control					
Ascorbic acid	5.1 ± 0.8	1.1 ± 0.03			
Propyl gallate				1.2 ± 0.01	1.0 ± 0.01
BHT			63.0 ± 4.1		

Data are expressed as means ± S.D. (n = 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α = 0.05): **** $p < 0.0001$, *** $p < 0.001$ compared with the positive controls.

Of interest is also the result obtained by this extract in FRAP test with a value of 71.5 μM Fe(II)/g, a better value than that obtained with the positive control BHT (63.0 μM Fe(II)/g) as well as inhibition of lipid peroxidation with an IC_{50} value of 8.5 μg/mL after 30 min of incubation, in the β-carotene bleaching test.

Methanol extract showed the most promising inhibition of lipid peroxidation evaluated by β-carotene bleaching test with an IC_{50} value of 6.6 μg/mL after 30 min of incubation and exhibited good activity in ABTS with an IC_{50} value of 22.5 μg/mL.

3.3. Cholinesterases Inhibitory Activity

In order to investigate the in vitro neuroprotective properties of Daphne laureola extracts, the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activity was tested by applying Ellman's method. The IC_{50} values and the selectivity index (SI) are reported in Table 2.

Daphne laureola extracts inhibited AChE and BChE in a concentration-dependent manner. The methanol extract was the most active against AChE (IC_{50} value of 56.7 μg/mL). *n*-Hexane extract was not active at the maximum tested concentration and dichloromethane showed a three times lower activity (IC_{50} value of 147.7 μg/mL).

An interesting result was obtained against BChE by the n-hexane extract (IC_{50} value of 49.6 μg/mL). The other two extracts inhibited BChE (IC_{50} values of 106.1 and 199.4 μg/mL for dichloromethane and methanol extracts, respectively).

Table 2. Acetylcholinesterase (AChE) and butyrylcholinetserase (BChE) inhibitory activity (IC_{50} μg/mL) of *Daphne laureola* extracts.

D. laureola	AChE	BChE	SI (BChE/AChE)
n-Hexane	NA	49.7 ± 2.1 ****	-
Dichloromethane	147.7 ± 4.4 ****	106.1 ± 4.3 ****	0.7
Methanol	56.9 ± 2.2 ****	199.4 ± 4.7 ****	3.5
Positive control			
Physostigmine	0.1 ± 0.01	0.2 ± 0.03	2.0

N.A.: Not active; Data are expressed as means ± S.D. (n = 3). Differences within and between groups were evaluated by one-way ANOVA followed by a multicomparison Dunnett's test (α = 0.05): **** $p < 0.0001$ compared with the positive controls.

4. Discussion

Examination of the brain of AD patients has revealed a great deal of oxidative damage, associated with both hallmark pathologies such as neurofibrillary tangles and senile plaques. This suggests that oxidative stress is an important event in AD pathogenesis. Many studies demonstrated the ability of natural compounds to slow the AD progression by acting as cholinesterase inhibitors and by protecting neurons from oxidative stress acting as antioxidant agents [11].

Various *Daphne* species are used in traditional medicine and exhibited several activities such as antimicrobial, anti-inflammatory, antiviral, antitumor, and antifertility activities [12–14].

In this work, *D. laureola* extracts were investigated as potential cholinesterases inhibitors and antioxidant agents by using different in vitro tests. This approach, based on the use of different assays, is mandatory because antioxidants can exert their capacity through different mechanisms of action. The most interesting results in the antioxidant assays were obtained with dichloromethane extract, followed by methanol extract. The first extract was particularly active as a radicals scavenging agent, the latter demonstrated a good inhibiting capacity of lipid peroxidation.

Other *Daphne* species have been studied as a source of antioxidant compounds. In our previous work, we investigated the antioxidant and anti-proliferative activities of *D. striata* and *D. mezereum* *n*-hexane, dichloromethane, and methanol extracts [15]. In agreement with our results, in the ABTS test, the dichloromethane extract of both species was the most active. *D. striata* methanol extract showed the strongest activity in the DPPH test. More recently, Tongur et al. [16] described significant lipid oxidation inhibiting capacity of *D. gnidioides* and *D. sericea* methanol extracts.

Instead, few studies in the literature have evaluated the potential neuroprotective activity of *Daphne* species [17,18]. Dapholosericol A and dapholosericin A isolated from the stems of *D. holosericea* exhibited moderate inhibitory effects with percentage inhibition of 36 and 29%, respectively, at the concentration of 100 μmol/L [17]. The methanol extract of *D. mucronata* showed a weak AChE inhibitory activity (8.2% at 300 μg/mL) [18].

Herein, *D. laureola* extracts exerted a promising cholinesterases inhibitory activity with IC_{50} value of 56.9 μg/mL against AChE for the methanol extract, and IC_{50} value of 49.7 μg/mL against BChE for the *n*-hexane extract. Cholinesterase inhibitors attenuate the cholinergic deficit underlying the cognitive and neurologic dysfunctions in AD patients. Inhibition of AChE has been the main therapeutic target for the treatment of AD. AChE-positive neurons project extensively to the cortex, modulating cortical processing and stimuli responses. BChE-positive neurons project precisely to the frontal cortex, and may have an important role in executive function, emotional memory, behaviour, and attention. Moreover, the activity of BChE gradually increases as the severity of disease advances, while the activity of AChE declines. So, inhibition of BChE may provide further benefits. AChE and BChE share approximately 65% amino acid sequence identity. Although closely related, these enzymes display different substrate specificities that only partially overlap. This disparity is generally due to differences in the number of aromatic amino acid residues

lining the active site gorge, which leads to large differences in the shape of the gorge and potentially to distinct interactions with an individual ligand [19,20].

5. Conclusions

In this work, *D. laureola* aerial parts collected in Southern Italy were studied. Our results suggest *D. laureola* extracts as a promising source of phytochemicals potentially useful for the treatment of neurodegenerative diseases such as AD. These findings provide the basis for additional in vivo studies that could support these in vitro results on the potential health benefits of *D. laureola* extracts and their pure constituents.

Author Contributions: R.T. and L.P. conceived and designed the experiments; M.B. and A.R.C. performed the experiments; M.R.L., R.T. and A.R.C. analyzed the data; L.P. contributed reagents/materials/analysis tools; R.T. and M.R.L. wrote the paper. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

The following abbreviations are used in this manuscript:

ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid
AChE	Acetylcholinesterase
BChE	Butyrylcholinesterase
BHT	Butylated hydroxytoluene
DPPH	2,2-Diphenyl-1-picrylhydrazyl
FRAP	Ferric Reducing Antioxidant Power
IC_{50}	Half Maximal Inhibitory Concentration
SD	Standard Deviation

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