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RESEARCH ARTICLE

Phytochemicals from *Dodonaea viscosa* and their antioxidant and anticholinesterase activities with structure–activity relationships

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ABSTRACT

Context *Dodonaea viscosa* (L.) Jacq (Sapindaceae) has been used in traditional medicine as antimalarial, antidiabetic and antibacterial agent, but further investigations are needed.

Objective This study determines the antioxidant and anticholinesterase activities of six compounds (**1**–**6**) and two crystals (**1A** and **3A**) isolated from *D. viscosa*, and discusses their structure–activity relationships.

Materials and methods Antioxidant activity was evaluated using six complementary tests, i.e., β-carotene-linoleic acid; $DPPH^{\bullet}$, $ABTS^{\bullet+}$, superoxide scavenging, CUPRAC and metal chelating assays. Anticholinesterase activity was performed using the Elman method.

Results Clerodane diterpenoids (**1** and **2**) and phenolics (**3**–**6**) – together with three crystals (**1A**, **3A** and **7A**) – were isolated from the aerial parts of *D. viscosa*. Compound **3A** exhibited good antioxidant activity in DPPH (IC_{50}: 27.44 ± 1.06 μM), superoxide (28.18 ± 1.35% inhibition at 100 μM) and CUPRAC ($A_{0.5}$: 35.89 ± 0.09 μM) assays. Compound **5** (IC_{50}: 11.02 ± 0.02 μM) indicated best activity in ABTS assay, and **6** (IC_{50}: 14.30 ± 0.18 μM) in β-carotene-linoleic acid assay. Compounds **1** and **3** were also obtained in the crystal (**1A** and **3A**) form. Both crystals showed antioxidant activity. Furthermore, crystal **3A** was more active than **3** in all activity tests. Phenol **6** possessed moderate anticholinesterase activity against acetylcholinesterase and butyrylcholinesterase enzymes (IC_{50} values: 158.14 ± 1.65 and 111.60 ± 1.28 μM, respectively).

Discussion and conclusion This is the first report on antioxidant and anticholinesterase activities of compounds **1, 2**, **5, 6**, **1A** and **3A,** and characterisation of **7A** using XRD. Furthermore, the structure–activity relationships are also discussed in detail for the first time.

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Clerodane diterpenoids; phenolics; phytochemistry; XRD study

Introduction

Antioxidants are common preservatives, and mostly synthetic antioxidants have been used to prevent oxidative deterioration in food and pharmaceutical industries. The use of antioxidants may slow the progress of Alzheimer's disease (AD) and minimise neuronal degeneration (Atta-ur-Rahman & Choudhary 2001) by inhibiting acetylcholinesterase enzyme. Since the only valid hypothesis being accepted is the lack or deficiency of acetylcholine (Grossberg 2003), acetylcholinesterase inhibitors are used to treat Alzheimer's disease (Scalbert et al. 2005). However, most of these drugs have side effects such as liver damage and bradycardia (Dökmeci 2000). Synthetic antioxidants also caused liver damage and carcinogenesis in rats (Grice 1988; Ito et al. 1983). That provoked scientists to search new natural and harmless antioxidants, as well as anticholinesterase compounds.

Dodonaea viscosa (L.) Jacq (Sapindaceae) is a flowering evergreen shrub. *Dodonaea* is a genus of 60 species that is widely distributed in warmer parts of Australia, South Africa, North America and South Asia countries (Abdullah 1973). Traditionally, *D. viscosa* has been used against skin diseases (Pirzada et al. 2010), and used as an antimalarial (Clarkson et al. 2004), antidiabetic (Ahmad et al. 2002) and antibacterial agent (Ahmad et al. 2002; Clarkson et al. 2004; Choudhary et al. 2013).

Owing to the pharmacological significance of *D. viscosa*, it was subjected to phytochemical investigations. Herein, the antioxidant and anticholinesterase activities of isolated compounds as well as their structure–activity relationships are reported. To the best of our knowledge, this is the first report of the antioxidant and anticholinesterase activities of isolated compounds (**1**, **2**, **5**, **6**, **1A** and **3A**). Crystal compound **7A** was also characterised using XRD for the first time.

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Materials and methods

General experimental procedure and chemicals

Bioactivity measurements were carried out on a 96-well microplate reader, SpectraMax 340PC^{384}, Molecular Devices (Silicon Valley, CA), at the Department of Chemistry, Muğla Sıtkı Kocman University. The measurements and calculations of the activity results were evaluated using Softmax PRO v5.2 software (Molecular Devices, Silicon Valley, CA). XRD analysis parameters were as follows: data collection, cell refinement and data reduction: CrysAlis PRO (Agilent 2010); structure solution and refinement software: SHELXS97 (Sheldrick 2008); molecular graphics: X-SEED (Barbour 2001); cif editor: publCIF (Westrip 2010); refinement: on F^2 full matrix least-squares.

Ethanol, *n*-hexane, methanol, ammonium acetate, copper (II) chloride, ferrous chloride, *N*-methyl-phenazoniummethylsulphate (PMS), nicotinamide adenine dinucleotide (NADH), nitrotetrazoliumblue chloride (NBT) and ethylenediaminetetraacetic acid (EDTA) were obtained from E. Merck (Darmstadt, Germany). β-Carotene, α-tocopherol, linoleic acid, polyoxyethylenesorbitanmonopalmitate (Tween-40), neocuproine, butylated hydroxyl anisole (BHA), 1,1-diphenyl-2-picrylhydrazyl (DPPH),3-(2-pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5′′-disulphonic acid disodium salt (Ferene), Tris-HCl, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), galantamine, acetylcholinesterase from electric eel (AChE, Type-VI-S, EC 3.1.1.7, 425.84 U/mg), butyrylcholinesterase from horse serum (BChE, EC 3.1.1.8, 11.4 U/mg), 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB), acetylthiocholine iodide and butyrylthiocholine chloride were obtained from Sigma Chemical Co. (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals and solvents were of analytical grade.

Plant material, extraction and isolation

Plant collection and voucher specimen information has been published previously (Muhammad et al. 2012a). The plant material (20 kg, shade-dried) was ground into powder and extracted at room temperature with 35 L MeOH three times for 15 days. After removing the solvent, the extract was suspended in H_2O and extracted with *n*-hexane, $CHCl_3$, EtOAc and *n*-BuOH to yield *n*-hexane (116 g), $CHCl_3$ (890 g), EtOAc (173 g) and *n*-BuOH (337 g) extracts, respectively. The crystals **1A** and **3A**, and compounds **1**–**6** were isolated and characterised according to previous reports (Anis et al. 2001; Mohammad et al. 2010, 2012b). Using NMR, MS and XRD, the purified compounds were elucidated as hautriwaic lactone (**1**), crystal of **1** (**1A**), 6β-hydroxy-15,16-epoxy-5β,8β,9β,10α-cleroda-3,13 (16),14-trien-18-oic acid (**2**), 3,4-dihydroxybenzoic acid (**3**), crystal of **3** (**3A**), vanillic acid (**4**), nebrodenside A (**5**) and docosyl caffeate (**6**). The structures of the isolated compounds (**1**–**7**) were given in Figure 1. The $CHCl_3$ fraction was subjected to MPLC [silica gel, hexane/EtOAc (1:0–0:1)] to obtain 23 fractions (Fr. A–W). Crystal of 15,16-epoxy-19-hydroxy-1,3,13-(16),14-cleroda-tetraen-18-oic acid (**7A**) (6.5 mg) was obtained from Fr. C as colourless crystals. Previously, **7A** was characterised by Ortega et al. (2001), although they did not obtain it in a crystal form. In this study, we obtained it in a crystal form. The stereochemistry of the **7A** had been ambiguous, hence we determined it through XRD for the first time (Figure 2).

Crystal data for compound 7A

$C_{20}H_{26}O_4$, M_W 330.28, orthorhombic, $P2_12_1$, $a = 6.7744$ (1) Å, $b = 14.0747$ (2) Å, $c = 9.5068$ (2) Å, $\alpha = 90.00°$, $\beta = 107.457$ (2)°, $\gamma = 90.00°$, $V = 864.70(3)$ Å^3, $Z = 2$, Mo Kα radiation, 3377 reflections, 225 parameters, $\mu = 0.701$ mm^{-1}, $T = 100$ K, $R = 0.0364$, $R_w = 0.0992$, $S = 1.066$. The XRD data for **7A** (Deposition no. CCDC1040933) have been deposited at the Cambridge Crystallographic Data Centre (Figure 2).

Determination of antioxidant activity

All activities were performed according to the standard literature procedures with slight modifications (Tel et al. 2012). Total antioxidant activity was evaluated using β-carotene-linoleic acid model test (Marco 1968). Free radical scavenging activity was determined spectrophotometrically by the DPPH assay (Blois 1958). The spectrophotometric analysis of $ABTS^{\bullet+}$ scavenging activity was determined according to the literature (Re et al. 1999). Superoxide anion radical scavenging activity was performed according to PMS-NADH-NBT method (Öztürk et al. 2014). CUPRAC antioxidant activity was performed according to Apak's procedure (Apak et al. 2004). Metal chelating activity of the compounds on Fe^{2+} was performed spectrophotometrically (Decker & Welch 1990).

Determination of anticholinesterase activity

Acetylcholinesterase and butyrylcholinesterase inhibitory activities were measured using the spectrophotometric method developed by Ellman et al. (1961) with slight modification (Tel et al. 2012).

Determination of IC_{50} and $A_{0.5}$ values

The results are expressed as 50% inhibition concentration (IC_{50}). The sample concentration that provides 50%

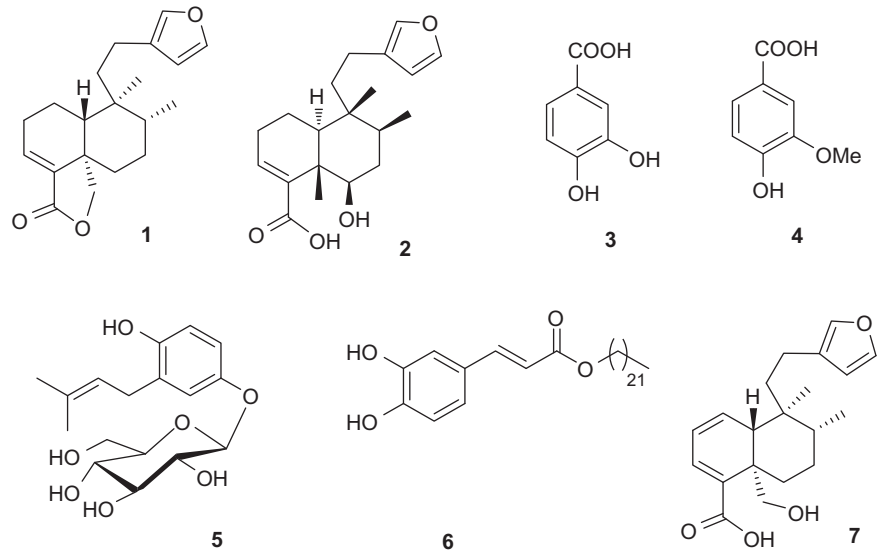

Figure 1. Structure of compounds isolated from *D. viscosa*.

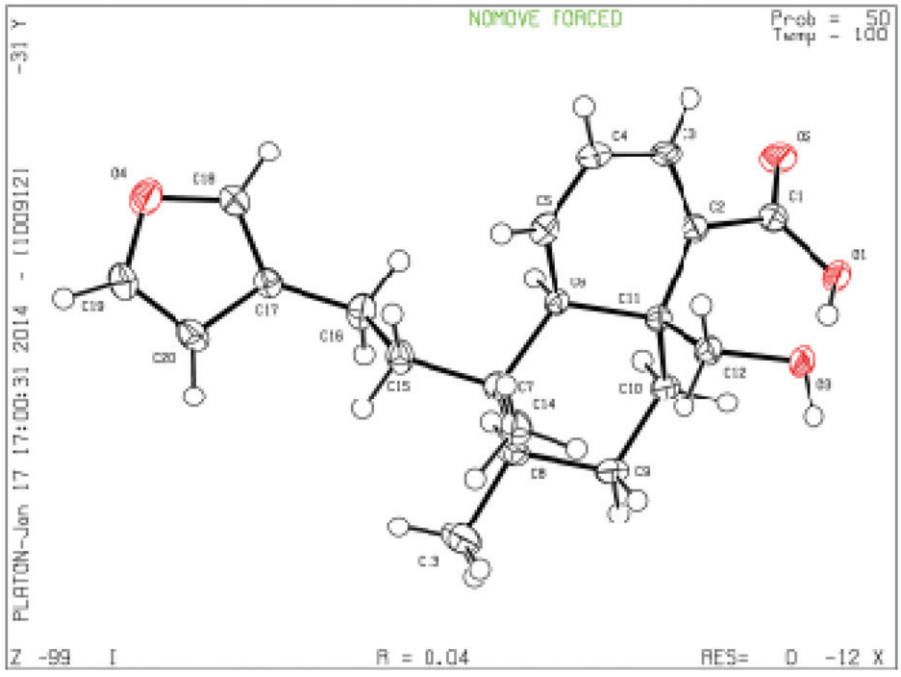

Figure 2. ORTEP drawing of **7A.**

activity (IC_{50}) was calculated from the graph of percent inhibitory activity versus sample concentration. The sample concentration having 0.50 absorbance ($A_{0.5}$) was calculated from the plot of CUPRAC absorbance against sample concentration.

Statistical analysis

The data of antioxidant and anticholinesterase activities were the averages of triplicate analyses. Data were recorded as mean ± standard error of mean (SEM). Significant differences between means were determined by Student's *t*-test, and p values <0.05 were considered as significant.

Results and discussion

Antioxidant activity

The antioxidant activity of diterpenes (**1, 2** and **1A**) and phenolics (**3–6** and **3A**) isolated from *Dodonaea viscosa* was tested using β-carotene-linoleic acid, DPPH radical scavenging, ABTS cation radical scavenging, superoxide

anion radical scavenging ($O^{2\bullet-}$), CUPRAC and metal chelating assays (Table 1). BHA, α-tocopherol and EDTA were used as positive standards. The tests were performed at different concentrations to calculate the IC_{50} and $A_{0.50}$ values. Results were statistically significant ($p < 0.05$) when compared with those of controls in each test.

The antioxidants and the lipid peroxidation inhibitors are easily tested in the β-carotene-linoleic acid assay, where $H^{\bullet}$ is transferred to the media by the antioxidant. Compound **6** exhibited the highest lipid peroxidation inhibitory activity (IC_{50}: 14.30 ± 0.18 μM), followed by **5** (IC_{50}: 40.30 ± 0.36 μM), **2** (IC_{50}: 107.45 ± 1.05 μM), **3A** (IC_{50}: 108.46 ± 1.08 μM) and **3** (IC_{50}: 147.45 ± 1.14 μM) (Table 1). Compounds **1, 1A** and **4**, however, showed weak inhibition activity (20.56 ± 1.09, 14.34 ± 0.40 and 5.42 ± 0.58% at 100 μM concentrations, respectively).

In $DPPH^{\bullet}$, $ABTS^{\bullet+}$ scavenging and CUPRAC assays, antioxidant transfers electron to the media. $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ are reduced by an electron or a radical species; thus, their absorbencies decrease at 517 and 734 nm, respectively. In CUPRAC assay, however, cupric is reduced to cuprous by an electron released by antioxidant. Then the cuprous forms a stable complex with neocuproine, which increases the absorbance at 450 nm. In $DPPH^{\bullet}$ assay, **3A** (IC_{50}: 27.44 ± 1.06 μM) exhibited better activity than α-tocopherol and very close activity to those of α-tocopherol and BHA, followed by **6** (IC_{50}: 78.54 ± 1.24 μM), **3** (IC_{50}: 166.06 ± 1.65 μM) and **5** (IC_{50}: 170.49 ± 1.76 μM). All other compounds exhibited weak DPPH radical scavenging activity (IC_{50} >200 μM). In $ABTS^{\bullet+}$ assay, **5** (IC_{50}: 11.02 ± 0.02 μM) exhibited the highest activity, even higher than that of α-tocopherol (IC_{50}: 17.18 ± 0.36 μM) and BHA (IC_{50}: 13.68 ± 0.54 μM). Compound **3A** (IC_{50}: 19.28 ± 0.56 μM) also indicated better activity which was very close to that of α-tocopherol. Compounds **3** (IC_{50}: 49.78 ± 0.19 μM) and **6** (IC_{50}: 51.61 ± 0.74 μM) demonstrated noticeable $ABTS^{\bullet+}$ scavenging activity as well. In CUPRAC assay, however, compound **3A** was the best reductant indicating 35.89 ± 0.09 μM $A_{0.50}$ value. At the same conditions, the $A_{0.50}$ values of BHA and α-tocopherol were 35.71 ± 1.68 and 40.48 ± 1.87 μM $A_{0.50}$, respectively. Compounds **3** ($A_{0.50}$: 55.78 ± 0.09 μM), **5** ($A_{0.50}$: 72.22 ± 0.21 μM) and **4** ($A_{0.50}$: 80.76 ± 0.08 μM) also exhibited good reducing capacity but less than those of antioxidant standards (Table 1).

The superoxide anion radical scavenging activity tests the superoxide quenching of the antioxidant. The superoxide generated by *N*-methyl-phenazonium-methylsulphate (PMS) from dissolved oxygen in NADH media. In this method, there is also electron transfer from antioxidant to quench the superoxide. The calculated IC_{50} values of compounds **1**–**6** were over 200 μM; thus, only the inhibition percentages at 100 μM are given (Table 1). The compounds (**3A** and **6**) indicating good antiradical activity also exhibited higher superoxide scavenging activities than the other compounds (**1**–**5** and **1A**). Herein, compound **2**, only active in β-carotene-linoleic acid assay, is interesting, and the activity can be explained by –OH at C-6 position (Table 1).

Metal chelating activity tests the secondary antioxidants that can bind the transition metals. Transition metals are the effective pro-oxidants, which accelerate the lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals via the Fenton Reaction (Halliwell & Gutteridge 1984). Iron is the typical example. Therefore, it is necessary to test the secondary antioxidants. Compounds **3** (26.55 ± 0.18%), **3A** (26.56 ± 0.04%) and **6** (25.18 ± 0.84%) possessed to chelate iron at 100 μM concentration.

Table 1. Antioxidant activity of the compounds of *Dodonaea viscosa* by the β-carotene-linoleic acid, $DPPH^{\bullet}$, $ABTS^{\bullet+}$, $O^{2\bullet-}$, CUPRAC and metal chelating assays[a].

		β-Carotene-linoleic acid assay IC_{50} (μM)	$DPPH^{\bullet}$ assay IC_{50} (μM)	$ABTS^{\bullet+}$ assay IC_{50} (μM)	$O_2^{\bullet-}$ assay Inhibition (%) (at 100 μM)	CUPRAC assay $A_{0.50}$(μM)	Metal Chelating assay Inhibition (%) (at 100 μM)
Compounds	**1**	>200	>200	>200	8.37 ± 1.23	>200	3.20 ± 0.65
	1A	>200	>200	>200	17.79 ± 0.01	>200	8.15 ± 0.22
	2	107.45 ± 1.05	>200	>200	23.98 ± 0.91	>200	9.64 ± 0.05
	3	147.45 ± 1.14	166.06 ± 1.65	49.78 ± 0.19	18.59 ± 0.41	55.78 ± 0.09	26.55 ± 0.04
	3A	108.46 ± 1.08	27.44 ± 1.06	19.28 ± 0.56	28.18 ± 1.35	35.89 ± 0.09	26.56 ± 0.18
	4	>200	>200	>200	14.36 ± 1.43	80.76 ± 0.08	3.96 ± 0.06
	5	40.30 ± 0.36	170.49 ± 1.76	11.02 ± 0.02	17.71 ± 1.12	72.22 ± 0.21	4.79 ± 0.54
	6	14.30 ± 0.18	78.54 ± 1.24	51.61 ± 0.74	26.48 ± 0.31	112.61 ± 0.42	25.18 ± 0.84
Standards	BHA[b]	1.03 ± 0.04	26.79 ± 0.65	13.68 ± 0.54	87.10 ± 0.92	35.71 ± 1.68	NT
	α-tocopherol[b]	2.08 ± 0.08	20.47 ± 0.57	17.18 ± 0.36	83.25 ± 1.41	40.48 ± 1.87	NT
	EDTA[b]	NT	NT	NT	NT	NT	94.43 ± 0.36

[a]IC_{50} values represent the means ± SEM of three parallel measurements ($p < 0.05$).
[b]Reference compounds.
NT: not tested; BHA: butylatedhydroxyl anisole; EDTA: ethylenediaminetetraacetic acid.

Anticholinesterase activity

Table 2 shows the acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibitory activities of the compounds (**1**–**6**, **1A** and **3A**) where galantamine was used as the standard. Compound **6** exhibited moderate activity against AChE and BChE enzymes (IC_{50}: 158.14 ± 1.65 and 111.60 ± 1.28 µM, respectively). Compound **1** (IC_{50}: 250.51 ± 1.54 µM) only indicated mild BChE inhibitory activity. According to Atta-ur-Rahman and Choudhary (2001), there is a correlation between antioxidant and anticholinesterase activities. However, only compound **6** followed this correlation in this study.

Structure–activity relationships

The skeleton of clerodane diterpenoids **1** and **2** is almost the same. Compound **1** possesses a lactone while **2** have two additional –OHs. Compound **1A** is the crystalline form of **1**. The diterpenes (**1**, **1A** and **2**) were tested for their potential antioxidant activity using six methods. Compound **2** exhibited noticeable lipid peroxidation inhibitory activity only in β-carotene-linoleic acid assay. The –OH at C-6 may be responsible for higher activity of **2** when compared with **1** or **1A** (Table 1).

The origin of antioxidant activity of phenolics is due to their hydroxyl groups. The location of the –OH groups can increase or decrease the activity. Particularly, the compounds having second –OH at *ortho* or *para* position exhibit higher antioxidant activity than at *meta* position. Effect of the functional group is responsible for antioxidant activity in the following order: –OH> –OAc> –C = O (oxo) (Farvin and Jacobsen 2013). In all antioxidant assays, the phenolic compounds (**3**, **3A**, **5** and **6**) exhibited good antioxidant activity. Compound **4** was active only in CUPRAC assay.

The structures of **3** and **4** are similar to each other. The difference is the replacement of – OH at C-3 position by the $-OCH_3$ group in **4**. However, the difference causes decrease in the antioxidant activity in case of $-OCH_3$ group.

Remarkably, all antioxidant activity tests revealed that the crystal (monohydrate) (**3A**) showed higher antioxidant activity than its amorphous form (**3**). On the other hand, anhydrous crystal **1A** showed close activity to its amorphous form (**1**). The reason for the higher activity is the presence of water in crystallisation in **3A**.

The antioxidant activity of phenolic compounds **5** and **6** is also comparable. Compound **5** has only one –OH group, and **6** has two *ortho* –OH groups at benzene ring. Because of these two *ortho* –OH groups, **6** exhibited better antioxidant activity than **5** in β-carotene, DPPH, superoxide and metal chelating assays. Interestingly, **5** exhibited better activity than **6** in ABTS and CUPRAC assays. The activity of **5** in ABTS and CUPRAC assays could be related to –OH group at C-1 position. The 3-methyl-2-butenyl group at C-2 position donates electrons to benzene ring; probably that is the reason for the better activity of **5**. Additionally, it is not easy for compound **5** to approach $DPPH^{\bullet}$ molecule due to its bulky 3-methyl-2-butenyl group. The structure–activity relationships showed that the position and number of –OHs can cause a tremendous effect on the biological activities.

Conclusion

Antioxidant and anticholinesterase activities of clerodane diterpenoids (**1** and **2**) and phenolics (**5** and **6**), isolated from *D. viscosa*, were determined for the first time. Unlike previous reports, compound **7A** was obtained in a crystal form and characterised by XRD for the first time. XRD structure confirmation also diminished the ambiguity in the stereochemistry of **7A**. It can be understood from the structure–activity relationship that the presence and position of –OH in the molecule is very important

Table 2. Anticholinesterase activity of the compounds of *Dodonaea viscosa*[a].

Anticholinesterase activity				
Compounds	AChE assay		BChE assay	
	Inhibition % at 100 µM	IC_{50} (µM)	Inhibition % at 100 µM	IC_{50} (µM)
1	26.29 ± 2.22	>400	45.57 ± 0.48	250.51 ± 1.54
1A	17.34 ± 1.53	>400	9.46 ± 0.03	>400
2	5.14 ± 0.05	>400	11.87 ± 0.08	>400
3	7.81 ± 0.04	>400	24.23 ± 1.22	>400
3A	12.65 ± 0.08	>400	25.52 ± 0.32	>400
4	13.13 ± 1.52	>400	38.97 ± 2.24	329.16 ± 2.01
5	22.16 ± 0.24	>400	11.97 ± 0.36	>400
6	40.36 ± 1.33	158.14 ± 1.65	47.78 ± 1.69	111.60 ± 1.28
Galantamine[b]	65.80 ± 1.08	4.48 ± 0.78	79.95 ± 0.86	46.03 ± 0.14

[a]IC_{50} values represent the means ± S.E.M. of three parallel measurements ($p < 0.05$).
[b]Reference compounds.
NT: not tested; NA: not active.

to give antioxidant activity. More –OH groups and its presence at *ortho* or *para* increases the biological activity, however, a *meta*-OH decreases. On one hand, compounds **3A** and **6** exhibited higher antioxidant activity in all tests while **5** in ABTS and in β-carotene-linoleic acid assays. Interestingly, monohydrate **3A** showed better antioxidant activity than amorphous **3**. On the other hand, amorphous **1** exhibited close activity to that of its crystal **1A**. It means that water of crystallisation can affect the antioxidant activity of a certain compound. We expect these compounds to have anticancer and enzyme inhibitory activities; similar to other antioxidants. Moreover, compound **6** was also found as a moderate inhibitor against AChE and BChE enzymes.

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Disclosure statement

The authors report that they have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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