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► To cite this version:

Wen Xu, Jianhua Wang, Baozhao Ju, Xiujuan Lan, Xixiang Ying, et al.. Seven compounds from *Portulaca oleracea* L. and their anticholinesterase activities. *Natural Product Research*, 2022, 36, pp.2547-2553. 10.1080/14786419.2021.1916928 . hal-03216986

HAL Id: hal-03216986

<https://hal.science/hal-03216986>

Submitted on 4 May 2021

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1 **Seven compounds from *Portulaca oleracea* L. and their anticholinesterase**
2 **activities**

3 Wen Xu ^{a, †}, Jianhua Wang ^{b, †}, Baozhao Ju ^b, Xiujuan Lan ^a, Xixiang Ying ^{a, *} and
4 Didier Stien ^{c, *}

5 ***Affiliation***

6 ^a School of Pharmacy, Liaoning University of Traditional Chinese Medicine, Dalian,
7 China

8 ^b School of Traditional Chinese Medicine, Liaoning University of Traditional Chinese
9 Medicine, Shenyang, China

10 ^c Sorbonne Université, CNRS, Laboratoire de Biodiversité et Biotechnologie
11 Microbiennes (LBBM), Observatoire Océanologique, Banyuls-sur-Mer, France

12 E-mail address in order:

13 xuwen199654@163.com, wjh-1985@163.com, jubaozhao@163.com,
14 xiujuanlan@139.com, yingxixiang@163.com, and didier.stien@cnrs.fr

15 ***Correspondence**

16 Correspondence author: Prof. Xixiang Ying, School of Pharmacy, Liaoning
17 University of Traditional Chinese Medicine, No.77, Shengming 1 Road, DD Port,
18 Dalian, 116600, P.R. China, Tel: +86-0411-85890139, Fax: +86-0411-85890128,
19 yingxixiang@163.com

20 Correspondence author: Dr. Didier Stien, Sorbonne Université, CNRS, Laboratoire
21 de Biodiversité et Biotechnologie Microbiennes, LBBM, Observatoire
22 Océanologique, 66650 Banyuls-sur-Mer, France, Tel: +33-430192476,
23 didier.stien@cnrs.fr

24 [†] Wen Xu and Jianhua Wang contributed equally to this work.

25

26 **Abstract**

27 A novel lignan, identified as
28 4-(3,4-dihydroxyphenyl)-6,7-dimethoxy-3a,4-dihydronaphtho[2,3-c]furan-1(3H)-one,
29 named oleralignan A (**1**), together with six known compounds, loliolide (**2**),
30 isolololide (**3**), dehydrolololide (**4**), daphnetin (**5**), esculetin (**6**), and trans-coumaric
31 acid methyl ester (**7**) was obtained from *Portulaca oleracea* L., while compounds **3**, **4**,
32 and **6-7** were isolated from the plant for the first time. Their structures were elucidated
33 using spectroscopic methods, including one- and two-dimensional NMR and
34 high-resolution electrospray ionization time-of-flight mass spectrometry. In addition,
35 the results of activity assay demonstrated that compounds **1-7** have anticholinesterase
36 activities.

37 **Keywords:** *Portulaca oleracea* L.; lignan; isolation; anticholinesterase activity

38

39 1. Introduction

40 *Portulaca oleracea* L., an annual green herb, belonging to the family of Portulacaceae,
41 with branched and succulent stems, is edible and has a slightly acidic and salty taste
42 similar to spinach (Lim and Quah 2007). It is consumed as a vegetable and has been
43 reported to provide better nutrition than the major cultivated vegetables (Yazici et al.
44 2007). It is widely distributed in Africa, China, India, Australia, Middle East, Europe
45 and America (Rashed et al. 2003). It has been listed by the World Health Organization
46 as one of the most used medicinal plants and has been given the term ‘Global
47 Panacea’ (Zheng et al. 2017). As a traditional Chinese medicine, it is well-known as
48 diuretic, febrifuge, antiseptic, antispasmodic and vermifuge (Shen et al. 2013). At the
49 same time, *P. oleracea* L. has been reported to possess a wide variety of
50 pharmacological effects, such as anti-inflammatory (Meng et al. 2016), antibacterial
51 (Nayaka et al. 2014), antiulcerogenic (Karimi et al. 2004), antitumor (Shen et al.
52 2013), antioxidant (Yang et al. 2019), antidiabetic (Bai et al. 2016), or
53 anticholinesterase (Xiu et al. 2019), among others. These activities have been related
54 to its chemical constituents including alkaloids (Zhao et al. 2019), flavonoids (Yang et
55 al. 2018), lignans (Ma et al. 2018), fatty acids (Teixeira et al. 2010), or minerals
56 (Uddin et al. 2012). In the present work, we isolated 7 interesting compounds from *P.*
57 *oleracea* L. including 1 new aryl-naphthalene lignan containing 2 methoxy groups. At
58 the same time, some lignans have been reported to have anticholinesterase activity,
59 and the methoxy group in lignans can effectively enhance its anticholinesterase effect
60 (Salleh et al. 2016). However, study on the anticholinesterase activity of
61 aryl-naphthalene lignans has not been found. Therefore, we undertook to evaluate their
62 anticholinesterase activities following the modified Ellman’s method.

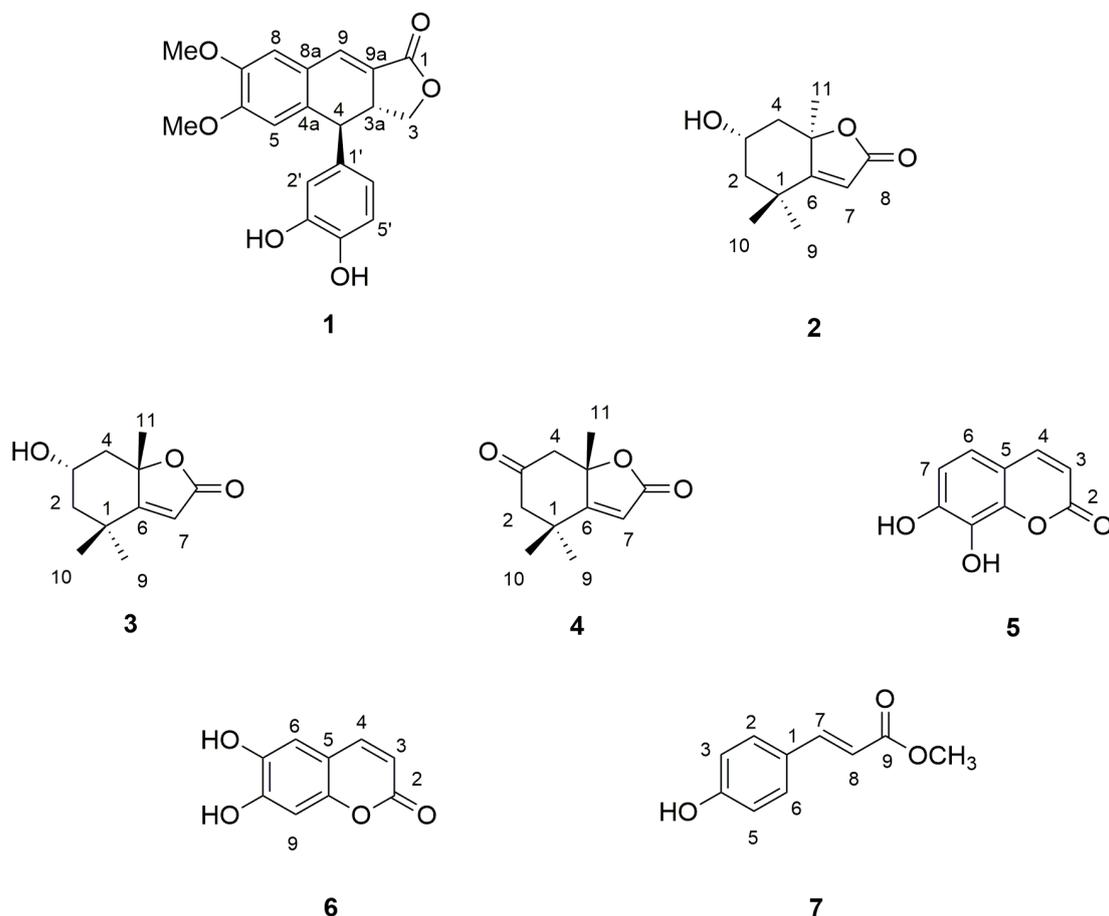
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64 2. Results and discussion

65 2.1. Structure elucidation

66 Compound **1**, a yellowish powder, has the molecular formula $C_{20}H_{18}O_6$, as deduced
67 from the HR-ESI-TOF-MS deprotonated molecular ion at m/z 353.1021 $[M-H]^-$
68 (calcd. for $C_{20}H_{17}O_6^-$, 353.1031), corresponding to 12 degrees of unsaturation. The
69 examination of the 1H , ^{13}C , and HSQC spectra of **1** demonstrated that several signals
70 were very broad in 1H and ^{13}C NMR spectra. Some signals were also duplicated. This
71 eventually turned out to originate from the slow rotation of the unsymmetrical phenyl

72 ring (1'-6'). This phenyl ring appears in ^1H NMR in the form of a very broad signal
73 between 6.70 and 6.94 ppm integrating for 3 protons. The signals of the methoxy
74 group in C-6 (δ_{H} 3.73, δ_{C} 55.6) and the methine 3a (δ_{H} 3.55, δ_{C} 39.7) are also very
75 broad due to the proximity of the rotating phenyl ring.
76



77

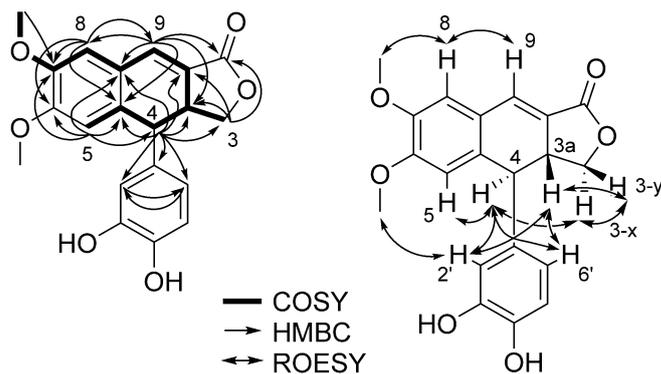
78 **Figure 1**

79

80 With regard to the structural determination itself, it was possible to establish the
81 presence of a 1,2,4,5-tetrasubstituted benzene ring based on the signals of protons H-5
82 (δ_{H} 6.16) and H-8 (δ_{H} 7.16) together with their respective carbons at δ_{C} 114.9 and
83 113.9 and their HMBC correlations with non-protonated carbons of the benzene ring
84 (**Table S1** in supplementary material, **Figure 2**). Since protons H-5 and H-8 correlated
85 with both C-6 and C-7, the assignment of both methoxy groups was based on the
86 COSY and ROESY correlations of MeO-C7 with H-8, but also the ROESY
87 correlation of MeO-C6 with H-2'. The COSY and ROESY spectra also provided key
88 information regarding the substituents in positions 4a and 8a. Thanks to these data, it

89 was possible to position H-4 close to H-5 and H-9 close to H-8. This was confirmed
 90 by the HMBC correlations of H-4 with C-4a and C-8a and of H-9 with C-4a, C-8a and
 91 C-8. The COSY spectrum defined a proton sequence H-4/H-3a/H-3, and the proton
 92 and carbon chemical shifts in position 3 (δ_{H} 3.97/4.33, δ_{C} 71.5) demonstrated that C-3
 93 was link to an oxygen. The HMBC correlations H-9/C-3a, H-9/C-1, and H-3-y/C-1
 94 completed the 3a,4-dihydroisobenzofuran-1-one subunit.

95



96

97 **Figure 2**

98

99 Additional correlations of H-4 with C-1', C-2' and C-6' demonstrated that an aromatic
 100 ring was linked to C-4 and that both *ortho* positions were protonated. The fact that the
 101 signals are very wide and are split does not prevent the structural determination
 102 because protons H-2' and H-6' also correlated in ROESY with H-3a, H-4 and for H-2',
 103 MeO-C6. As the lateral aromatic ring could not be symmetrical, two hydroxy groups
 104 had to be positioned on the C-3' and C-4' carbons. The hydroxy protons are visible in
 105 ^1H NMR at δ_{H} 8.97 and 9.45. The remaining methine was assigned to position 5'. The
 106 relative configuration of compound **1** was ascertained based on the 8.6 Hz coupling
 107 between H-3a and H-4 along with the ROESY correlations H-4/H-3-x and H-3a/H-3-y
 108 which were consistent with an anti-periplanar configuration of protons H-3a and H-4.
 109 Coupling constants of 8-10 Hz are found in analogous compounds described in the
 110 literature, including conidendrin (Barton 1968; Davies and Jin 2003). Eventually,
 111 compound **1** was identified as a conidendrin-related lignan and was given the trivial
 112 name oleralignan A.

113 By comparison of the ^1H and ^{13}C NMR spectral data with literature, the 6 known
 114 compounds (**2-7**) were identified, respectively, as loliolide (**2**) (Mori and Khlebnikov
 115 1993), isololiolide (**3**) (Zheng et al. 2014), dehydrololiolide (**4**) (Zheng et al. 2014),

116 daphnetin (**5**) (Xu et al. 2008), esculetin (**6**) (Zhou et al. 2009), trans-coumaric acid
117 methyl ester (**7**) (Wan et al. 2012).

118 **2.2. Anticholinesterase activity**

119 Alzheimer's disease (AD) is the most common neurodegenerative disorder of aging,
120 accounting approximately for two-third of all cases of senile dementia (Tandon et al.
121 2000). AD is neurochemically characterised by a consistent deficit in cholinergic
122 neurotransmission, particularly affecting cholinergic neurons in the basal forebrain.
123 Symptoms of AD and other forms of dementia can be treated by the use of agents
124 which restore the level of acetylcholine through inhibition of both major
125 cholinesterases: acetylcholinesterase and butyrylcholinesterase (Loizzo et al. 2009).
126 Studies have shown that some lignans exhibit neuroprotective effects, increasing
127 choline acetyltransferase activity, inhibiting acetylcholinesterase (AChE) activity *in*
128 *vitro*, and increasing hippocampal acetylcholine release *in vivo* (Howes and Houghton
129 2003). Consequently, the anticholinesterase activities of the 7 compounds were
130 evaluated using a microplate assay. Oleralignan A (**1**) inhibited enzyme activity at
131 $58.31 \pm 0.23 \mu\text{M}$. Compounds **2-7** activity ranged from $71.50 \pm 0.39 \mu\text{M}$ to $93.88 \pm$
132 $0.39 \mu\text{M}$. Overall, oleralignan A exhibited higher anticholinesterase activity than
133 compounds **2-7**(**Table S2**, in supplementary material).

134 **3. Experimental**

135 **3.1. General experimental procedures**

136 The NMR spectra were obtained using an AVANCE 600 MHz instrument (Bruker
137 Corporation, Switzerland). HRMS was determined using a 6520
138 quadrupole-time-of-flight mass spectrometer (Agilent, Palo Alto, CA). The UV data
139 was obtained from a HITACHI U-3010 spectrophotometer (Hitachi Ltd, Tokyo,
140 Japan), the IR spectrum and data obtained from IR200 spectrophotometer (Thermo
141 Electron Corporation, Waltham, MA). In the separation process, the GF₂₅₄ (Qingdao
142 Marine Chemical Co., Qingdao, China) was being as TLC filling, the silica gel
143 (100-200 and 200-300 mesh, Qingdao Marine Chemical Co., Qingdao, China),
144 polyamide resin (80-100 mesh, Taizhou Luqiao Sijia Biochemical Plastic Factory,
145 Zhejiang, China) and ODS (20-40 μm , GE Healthcare, Marlborough, MA) were used
146 as column chromatographic filling. The 96-well microplate reader (HBS-1096A) was
147 from Nanjing Detie Experimental Equipment Co., Ltd. (Nanjing, China).

148 **3.2. Plant materials and chemicals**

149 The whole herbs of *P. oleracea* L. were collected in Shijiazhuang (Hebei, China) in
150 June 2017, and identified by Prof. Xixiang Ying. The voucher specimens (No.
151 20171001) were deposited at School of Pharmacy, Liaoning University of Traditional
152 Chinese Medicine. Acetylthiocholine iodide (ATCI) (purity \geq 99%) and AChE
153 (vitality \geq 200 units/mg protein) were from Dalian Meilun Biotechnology Co., Ltd.
154 (Dalian, China), 5, 5-dithiobis-2-nitrobenzoic acid (DTNB) (purity \geq 99%) was from
155 Shanghai Jinsui Bio-Technology Co., Ltd. (Shanghai, China) and eserine (purity \geq
156 98%) was from Shanghai Hanxiang Biotechnology Co., Ltd. (Shanghai, China). The
157 HPLC grade formic acid, acetonitrile and methanol were purchased from Damao
158 Chemical Reagent Plant (Tianjin, China, purity \geq 99.9%). All other reagents were
159 analytical grade reagents supplied by Jinfeng Chemical Factory (Tianjin, China), and
160 the purified water was from WAHAHA Company (Shenyang, China).

161 **3.3. Isolation and identification**

162 The dried whole herb of *P. oleracea* L. (250 kg) were extracted twice with 10
163 volumes of water for 2 hours each time, and then the water extract was concentrated
164 to obtain a crude extract (21 kg), which was subjected to a 100-200 mesh silica-gel
165 column chromatography (61 \times 55 cm, approximately, 70 kg) with a 2 volumes of
166 ethyl acetate 3 times as the isocratic elution, affording the fraction evaporated (550 g).
167 Then, the fraction was subjected to the 80-100 mesh polyamide resin column
168 chromatography (120 \times 8 cm, approximately, 1 kg), eluting with water, 30%, 50%,
169 70% and 100% ethanol to obtain five fractions (4 L each). The fraction of 50%
170 extracting (121 g) was condensed then subjected to a 200-300 mesh silica-gel column
171 (120 \times 8 cm, approximately, 1 kg) for further purification with ethyl acetate, ethyl
172 acetate and methanol (5:1, 2:1, v/v) as the gradient eluant, obtaining three fractions
173 (Frs. 1-3, 4 L each). The fraction of ethyl acetate (69 g) was subjected to the 20-40 μ m
174 octadecylsilyl (ODS) column chromatography (25 \times 3 cm, approximately, 150 g,
175 Ultimate XB-C18) with methanol and water (60:40, 70:30, 80:20, 90:10, 100:0, v/v)
176 as the gradient eluant under medium pressure, acquiring five fractions (A1-A5, 500
177 mL each). Among that, A1 was purified on a Sephadex LH-20 column (100 g, ϕ 2 \times
178 150 cm) using methanol solvent to obtain seven fractions B1-B7, and then these
179 fractions were further prepared by UHPLC, using MeOH-0.1% formic acid as the
180 mobile phase, with flow rate of 1.0 mL/min, and obtained compound **1** (2 mg, purity
181 of $>$ 98% with UHPLC, t_R 13.82 min, MeOH-0.1% formic acid, 61:39, v/v),

182 compound **2** (2 mg, purity of > 98% with UHPLC, t_R 25.32 min, MeOH-0.1% formic
183 acid, 20:80, v/v), compound **3** (1 mg, purity of > 98% with UHPLC, t_R 18.14 min,
184 MeOH-0.1% formic acid, 20:80, v/v), compound **4** (3 mg, purity of > 97% with
185 UHPLC, t_R 17.67 min, MeOH-0.1% formic acid, 20:80, v/v), compound **5** (1 mg,
186 purity of > 98% with UHPLC, t_R 15.32 min, MeOH-0.1% formic acid, 12:88, v/v),
187 compound **6** (2 mg, purity of > 98% with UHPLC, t_R 19.14 min, MeOH-0.1% formic
188 acid, 10:90, v/v), compound **7** (3 mg, purity of > 98% with UHPLC, t_R 9.76 min,
189 MeOH-0.1% formic acid, 45:65, v/v).

190 Oleralignan A (**1**): Yellowish powder; UV (CH₃OH) λ_{max} 348, 254 nm; IR (KBr) ν_{max}
191 2934, 2850, 1740, 1565, 1518, 1452, 1212 cm⁻¹; ¹H NMR (600 MHz, DMSO-*d*₆) and
192 ¹³C NMR (150 MHz, DMSO-*d*₆), see **Table S1** (in supplementary material);
193 HR-ESI-MS m/z 353.1021 [M-H]⁻ (calcd. for C₂₀H₁₇O₆⁻, 353.1031).

194 **3.4. Anticholinesterase activity assay**

195 Acetylcholinesterase activities of compounds were assessed by a modified Ellman's
196 method (Ellman et al. 1961). In a 96-well plate, 140 μ L PBS (0.1 M, pH = 8.0,
197 containing 0.1 mol/L NaH₂PO₄ and Na₂HPO₄), 10 μ L DTNB (15 mmol/L), 15 μ L
198 AChE (0.2 U/mL) and 20 μ L of sample solution serially diluted with methanol were
199 mixed and incubated at 37°C for 10 minutes, then 10 μ L ATCI (15 mmol/L) was
200 added and incubated at 20°C for 10 minutes. The absorbance was measured at 405 nm.
201 Eserine and methanol were used as positive and blank controls, respectively. The
202 inhibition rate (%) of each sample AChE was calculated according to the following
203 formula (Aktumsek et al. 2013): Inhibition activity (%) = [(A_{blank} - A_{sample})/A_{blank}] ×
204 100%, in which, A_{sample} and A_{blank} were respectively the absorbance of the test
205 compound and the blank control. The anticholinesterase activity was evaluated by the
206 value of IC₅₀ (means ± SD, $n = 3$).

207 **4. Conclusion**

208 In the study, a novel lignan named oleralignan A and six known compounds were
209 isolated from the water extract of *P. oleracea* L. The results of anticholinesterase
210 activities assay showed that oleralignan A presented a higher anticholinesterase
211 activity than others, with IC₅₀ value of 58.31 ± 0.23 μ M.

212 **Supplementary material**

213 Supporting information can be found in the online version of this article.

214 **Disclosure statement**

215 The authors have no conflicts of interest to disclose.

216 **Acknowledgements**

217 This work was supported by the National Natural Science Foundation of China (grant
218 number 82073990).

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