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1 **An isoindole alkaloid from *Portulaca oleracea* L.**

2 Mingyue Jiang^a, Wenjie Zhang^a, Xu Yang^a, Fen Xiu^a, Haoran Xu^a, Xixiang Ying^{*a},
3 Didier Stien^{*b}

4 ^a *School of Pharmacy, Liaoning University of Traditional Chinese Medicine, 77*
5 *Shengming 1 Road, DD Port, Dalian 116600, China*

6 ^b *Sorbonne Universités, UPMC Univ Paris 06, CNRS, Laboratoire de Biodiversité et*
7 *Biotechnologies Microbiennes (LBBM), Observatoire Océanologique, Banyuls-sur-*
8 *Mer, France*

9

10 *Corresponding author. E-mail: yingxixiang@163.com and didier.stien@cnrs.fr

11 Corresponding author: Prof. Xixiang Ying

12 Email: yingxixiang@163.com

13 Affiliation: School of Pharmacy, Liaoning University of Traditional Chinese Medicine

14 Address: No. 77, Shengming 1 Road, DD Port, Dalian, P.R. China

15 Address code: 116600

16 Tel.: +86-0411-85890139

17 Fax: +86-0411-85890128

18 Corresponding author: Prof. Didier Stien

19 Email: didier.stien@cnrs.fr

20 Affiliation: Sorbonne Universités, UPMC Univ Paris 06, CNRS, Laboratoire de
21 Biodiversité et Biotechnologies Microbiennes (LBBM)

22 Address: Observatoire Océanologique, Banyuls-sur-Mer, France

23 Address code: 66650

24 Tel: +33-430192476

25 **ABSTRACT**

26 A novel isoindole alkaloid named oleraisoindole (**1**), together with six known
27 compounds, 7'-ethoxy-*trans*-feruloyltyramine (**2**), *N-trans*-feruloyltyramine (**3**), *N-*
28 *trans*-feruloyl-3-methoxytyramine (**4**), *N-trans-p*-coumaroyltyramine (**5**)
29 aurantiamide (**6**) and ferulic acid methyl ester (**7**) were isolated from *Portulaca*
30 *oleracea* L.. Compounds **2** and **7** were isolated for the first time from this plant.
31 Compounds **1** was identified using spectroscopic methods including HR-ESI-TOF-MS,
32 1D-NMR, 2D-NMR. It was tested in a nitric oxide (NO) inhibition assay and was
33 shown to inhibit NO production in RAW 264.7 cells induced by LPS.

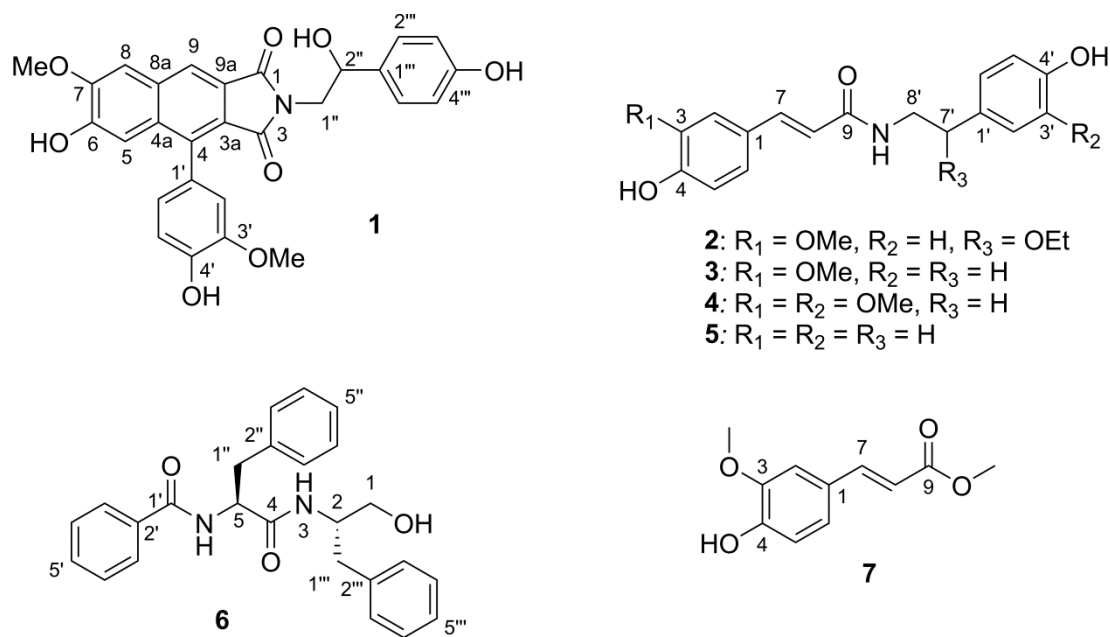
34 **KEYWORDS:** *Portulaca oleracea* L.; alkaloid; NO

35

36 **1. Introduction**

37 *Portulaca oleracea* L., as a well-known traditional Chinese medicine was recorded in
38 the Chinese Pharmacopoeia (The Pharmacopoeia Commission of PRC. 2015), presents
39 many biological and pharmacological activities such as anticancer (Zhao, et al. 2013),
40 anti-inflammatory and anti-oxidation (Yang, et al. 2016), anti-diabetic (Liang, et al.
41 2014) and lowering blood lipids (Zidan, et al. 2014). Thus, many scholars paid more
42 attention on the constituents of *P. oleracea* such as alkaloids (Jiao, et al. 2015, Xiang,
43 et al. 2005), flavonoids (Xu, et al. 2006), terpenes (Elkhayat, et al. 2008),
44 polysaccharides (Zhao, et al. 2015), and so on. Recently, many alkaloids were isolated
45 from *P. oleracea*, and some were found to have a very good anti-inflammatory effect
46 and other bioactivities (Li, et al. 2016, Li, et al. 2017, Li, et al. 2017, Meng, et al. 2016,
47 Xu, et al. 2016).

48 As the alkaloids isolated from this plant presented higher activities than the other
49 compounds, we embarked upon finding more alkaloids in *P. oleracea*. A novel
50 isoindole alkaloid, named oleraisoindole (**1**) and six known compounds, including 7'-
51 ethoxy-*trans*-feruloyltyramine (**2**) (Maciel, et al. 2015), *N-trans*-feruloyltyramine (**3**)
52 (Tian, et al. 2014), *N-trans*-feruloyl-3-methoxytyramine (**4**) (Kokubun, et al. 2012),
53 *N-trans*-p-coumaroyltyramine (**5**) (Kokubun, et al. 2012), aurantiamide (**6**) (Xu, et al.
54 2010), ferulic acid methyl ester (**7**) (Chen, et al. 2012) were finally isolated from the
55 water extract from *P. oleracea*. The structure of compound **1** was determined by HR-
56 ESI-TOF-MS, 1D-NMR, 2D-NMR and other compounds were identified by
57 comparison with literature data (Figure 1). In addition, we also investigated the
58 potential of compound **1** to inhibit the NO production in LPS-stimulated RAW 264.7
59 cells.



60

61 **Figure 1.** Chemical structures of compounds isolated from *P. oleracea*

62

63 2. Results and discussion

64 Compound **1** was a pale yellow powder, which showed a blue fluorescence spot at
 65 UV 365 nm, a black spot at UV 254 nm and remained orange when exposed to
 66 Dragendorff reagent on a TLC plate. UV (MeOH) λ_{max} : 284 nm (Figure S3). IR (KBr)
 67 ν_{max} : 3425, 1753, 1700, 1515, 1390, 1271, 1216 cm^{-1} (Figure S4). Its molecular
 68 formula $\text{C}_{28}\text{H}_{23}\text{NO}_8$ with 18 degrees of unsaturation was deduced from the ^1H -NMR,
 69 ^{13}C -NMR spectrum data (Table S1) and the HR-ESI-TOF-MS deprotonated
 70 molecular ion at m/z 500.1374 (calcd. 500.1351), along with the $[\text{M}-\text{H}_2\text{O}+\text{H}]^+$ ion at
 71 m/z 484.1397 in HR-ESI⁺-TOF-MS (calcd. 484.1391) (Figure S5(a)(b)). At first sight,
 72 this compound appeared as a mixture of two very closely related molecules because
 73 some signals are doubled both in ^1H and in ^{13}C NMR spectra (Table S1-S2). We
 74 pursued with the structural determination considering each doubled peaks as one signal
 75 hoping that the structure would eventually enlighten us on the origin of the doubled
 76 signals.

77 The examination of NMR data allowed to point out two phenyl rings. The HMBC
 78 spectrum showed the correlations from H-2', to C-4, C-4', C-6'; from H-5' to C-1', C-

79 3', C-4', C-6'; from H-6' to C-4, C-1', C-4', C-5', together with the ¹H-¹H COSY
80 correlations from H-5' to H-6' suggesting that protons at δ 6.84/6.93, 6.75/6.78, and
81 6.95 defined a 1,3,4-trisubstituted phenyl moiety, which was linked to carbon C-4 at
82 δ 139.6. This phenyl ring bore a methoxy group in C-3' and a hydroxyl in C-4'. The
83 position of the methoxy group could be inferred based on HMBC correlation H-5'/C-
84 3' and was confirmed by the NOE correlation of the methyl with H-2'. The ¹H-¹H
85 COSY spectrum showed the correlation H-2'''/H-6''', H-3'''/H-5''', and in HMBC the
86 equivalent proton H-2'''/H-6''' correlated to C-2'', C-2'''/C-6''' and C-4'''; H-3'''/H-5''' to
87 C-1''', C-3'''/C-5''', C-4'''. These are typical of a *para*-disubstituted phenyl ring. A
88 hydroxyl could be placed in 4''' based on C-4''' chemical shift (δ 158.2). The H-2'''/C-
89 2'' HMBC correlation indicated that C-2'' was linked to C-1'''. The ¹H and ¹³C chemical
90 shifts in position 1'' (δ _H3.67/3.70 and 3.88, δ _C46.1/46.2) are typical of a CH₂ linked to
91 a nitrogen, while C-2'' bore a hydroxyl (δ _H4.93, δ _C71.8/72.0). In HMBC, H-1''
92 correlated to carbonyl groups C-1 and C-3 at δ 170.2 and 169.1/169.3, respectively. In
93 ¹H-NMR, only four signals remained to be attributed. These corresponded to a methyl
94 at δ _H4.04, δ _C56.55 and three singlet CH at δ _H8.17 (H-9), 7.53 (H-8), and 7.15 (H-5),
95 with respective carbons at δ _C123.30, 110.0, and 112.1. This group also contained nine
96 quaternary carbons. These include C-4 at δ 139.6 and carbonyls C-1 and C-3, which
97 are linked to the already established peripheral groups. Protons H-8 and H-9 were not
98 coupled to each other. Nevertheless, they correlated in NOE indicating that they were
99 close to each other but not attached to a single phenyl ring. Also, H-8 correlated to C-
100 9 and H-9 to C-8 in HMBC. Altogether, these indications led to position two *peri*
101 protons on a naphthalene moiety. In HMBC, H-8 correlated to C-4a, C-6, C-7, and C-
102 9. It was not possible to be sure of the position of the methoxyl and the hydroxyl based
103 on HMBC only. However, strong NOE correlation between the methyl group and H-
104 8 allowed to place the methoxyl substituent in C-7. H-9 correlated to C-8 and C-4a
105 which further confirmed the relative place of H-8 and H-9. H-9 also correlated to C-1
106 and C-3a. It was therefore possible to link C-1 to C-9a. The C-3–C-3a bond could not
107 be formally detected, but it was reasonable to link these carbons based on chemical
108 shift of quaternary carbon C-3a (δ 123.29). Since the last singlet proton H-5 at δ 7.15
109 correlated to both C-6 and C-7, it had to be placed in *para*-position with respect to H-
110 8. H-5 also correlated to C-8a and C-4. The latter established the position of the
111 tetrasubstituted phenyl ring, which was confirmed by the NOE correlation between H-

112 5 and H-6'. C-9a could be detected neither in ^{13}C , nor in HMBC. NMR experiments
113 were also conducted in DMSO- d_6 . In this solvent, the ^{13}C NMR spectrum was of better
114 quality and the only peak that did not correlate in HMBC was at $\delta 125.20/125.23$. This
115 signal was attributed to C-9a. Compound **1** was determined to be the 6-hydroxy-2-(2-
116 hydroxy-2-(4-hydroxyphenyl)ethyl)-4-(4-hydroxy-3-methoxyphenyl)-7-methoxy-
117 1*H*-benzo[*f*]isoindole-1,3(2*H*)-dione, named oleraisoindole. The optical rotation of **1**
118 was found to be -6° (0.1, MeOH). One center of atropoisomerism (the C-4–C-1' bond)
119 accounts for the duplication of many NMR signals. The relative proportion of the two
120 rotamers as measured on the 3'-OMe proton signals is about 1:1.

121 The structures of six known compounds were identified based on the ^1H and ^{13}C
122 NMR spectra (Figure S20-31), and comparison with literature data, as 7'-ethoxy-*trans*-
123 feruloyltyramine (**2**), *N-trans*-feruloyltyramine (**3**), *N-trans*-feruloyl-3-
124 methoxytyramine (**4**), *N-trans-p*-coumaroyltyramine (**5**) aurantiamide (**6**) and ferulic
125 acid methyl ester (**7**).

126 From the MTT assay (Figure S32), compound **1** presented cytotoxicity at the
127 concentration of $100\ \mu\text{M}$. The concentrations of 1 to $50\ \mu\text{M}$ were therefore selected for
128 the NO assay. Compound **1** significantly inhibited NO production at $20\ \mu\text{M}$ on wards
129 (Figure S33).

130 **3. Experimental**

131 **3.1 General Experimental Procedures**

132 NMR spectra were recorded on an AVANCE 500 MHz instrument (Bruker
133 Corporation, Switzerland). UV spectra were detected on a U-3010 spectrophotometer
134 (Hitachi Ltd, Tokyo, Japan). IR spectra were obtained on an IR200 spectrophotometer
135 (Thermo Electron Corporation, Waltham, MA). MS spectra were measured on an
136 Agilent 6520 quadrupole-time of flight mass spectrometer (Agilent, Waldbronn,
137 Germany). Purification was measured with A Shimadzu Nexera X2 UHPLC LC-30A
138 system (Shimadzu, Kyoto, Japan). Optical rotation was detected on an autopol I
139 automatic polarimeter (Rudolph Research Analytical, Hackettstown, NJ). Sephadex
140 LH-20 and ODS (40-70 μm mesh, GE Healthcare, Marlborough, MA) were used for

141 Column chromatography (CC). TLC was conducted on silica gel GF₂₅₄ (Qingdao
142 Marine Chemical Co., Qingdao, China).

143 **3.2 Plant materials**

144 The dried plants of *P. oleracea* were collected in Shijiazhuang, Hebei, China in June
145 2014 and were identified by Xixiang Ying. A voucher specimen (No. 20140312) was
146 deposited in our laboratory at Liaoning University of Traditional Chinese Medicine.

147 **3.3 Extraction and isolation**

148 The dried plants (150 kg) of *P. oleracea* L. were extracted with 10 fold of hot water
149 twice times, each time for 2 h. The water extract was concentrated and then extracted
150 with 1 fold of ethyl acetate three times. The ethyl acetate extract was concentrated
151 under reduced pressure to obtain crude extract (200 g) and then subjected to
152 chromatography on a silica-gel (200-300 mesh, about 1.5 kg) column ($\phi 8 \times 120$ cm)
153 with ethyl acetate and methanol as the gradient eluent (3:1, 1:1, 1:3, v/v) to obtain 20
154 fractions (400 mL each). The fractions of 1-2 were combined and repeatedly
155 chromatographed by a 20-40 μ m ODS-C₁₈ (100 g) column ($\phi 3 \times 70$ cm) with methanol
156 and water as the gradient eluent (40:60, 60:40, 80:20, 100:0, v/v) to obtain 11 fractions
157 (200 mL each). The fractions of 1-2 were separated by a Sephadex LH-20 (100 g)
158 column ($\phi 2 \times 150$ cm) with methanol-water (70:30, v/v) as the eluent to obtain 42
159 fractions (20 mL each). The fractions of 26-30 were purified with UHPLC and eluted
160 with acetonitrile and water (30:70, v/v, 1.0 ml/min), affording oleraisoindole (**1**) (15
161 mg, purity > 98% with UHPLC), together with six known compounds, 7'-ethoxy-
162 *trans*-feruloyltyramine (**2**) (20 mg, purity > 98% with UHPLC), *N-trans*-
163 feruloyltyramine (**3**) (15 mg, purity > 98% with UHPLC), *N-trans*-feruloyl-3-
164 methoxytyramine (**4**) (10 mg, purity > 98% with UHPLC), *N-trans-p*-
165 coumaroytyramine (**5**) (8 mg, purity > 98% with UHPLC), aurantiamide (**6**) (20 mg,
166 purity > 98% with UHPLC) and ferulic acid methyl ester (**7**) (30 mg, purity > 98%
167 with UHPLC). Compound **1-6** turned orange when sprayed with Dragendorff reagent
168 on thin-layer chromatography.

169 Oleraisoindole (**1**): pale yellow powder, $[\alpha]_D^{20}$ -0.6 (0.1, MeOH). UV (MeOH) λ_{\max} :
170 284nm. IR (KBr) ν_{\max} : 3425, 1753, 1700, 1515, 1390, 1271, 1216 cm^{-1} . ¹H NMR and

171 ¹³C NMR spectral data (in methanol-*d*₄ and DMSO-*d*₆), see Table S1. HR-ESI(+)-
172 TOF-/MS m/z 484.1397 [M-H₂O+H]⁺ (calcd: C₂₈H₂₂NO₇, 484.1391). HR-ESI(-)-
173 TOF-/MS m/z 500.1374 [M- H]⁺ (calcd: C₂₈H₂₂NO₈, 500.1351).

174 **3.4 Biological assays**

175 The RAW 264.7 macrophage cell was incubated in DMEM containing 10% heat
176 inactivated fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin.
177 Cells were seeded in 96-well plates at a density of 1 × 10⁴ cells per well. Cell viability
178 was performed using MTT assay. The cells were incubated with various concentrations
179 (1, 10, 20, 50 and 100 μM) of oleraisoindole for 1 h, then 5 mg/ml MTT solution was
180 added after stimulated with LPS (1 μg/mL) for 24 h and incubated for another 4 h at
181 37°C. The formazan was dissolved in 150 μl of DMSO. The absorbance was measured
182 at 570 nm using a Bio-Tek microplate reader, cell viability was expressed as a
183 percentage by comparing with the untreated cells. The production of NO was measured
184 by Griess reaction. An equal volume of Griess reagent (0.1% naphthylethylethylene
185 diamine dihydrochloride in H₂O and 1% sulphanilamide in 5% H₃PO₄) was added to
186 the different concentrations of oleraisoindole cell culture supernatant (100 μl) collected
187 before add MTT solution in MTT assay. The production of NO was detected at 550
188 nm by a Bio-Tek microplate reader, and was compared with sodium nitrite standard
189 calibration curve.

190 **4. Conclusion**

191 A novel isoindole alkaloid named oleraisoindole (**1**) and six known compounds were
192 isolated from *P. oleracea*. Their structures were determined by spectroscopic analysis
193 techniques and comparison with literature data. The new alkaloid has unique chemical
194 structure and remarkably inhibiting NO production in RAW 264.7 cells induced by
195 LPS.

196 **Disclosure statement**

197 No potential conflict of interest was reported by the authors.

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204 **Supplementary material**

205 Supplementary material relating to this article is available online, alongside Tables S1-
206 S2 and Figure S1-S33.

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