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ABSTRACT

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

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

1. Introduction

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

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

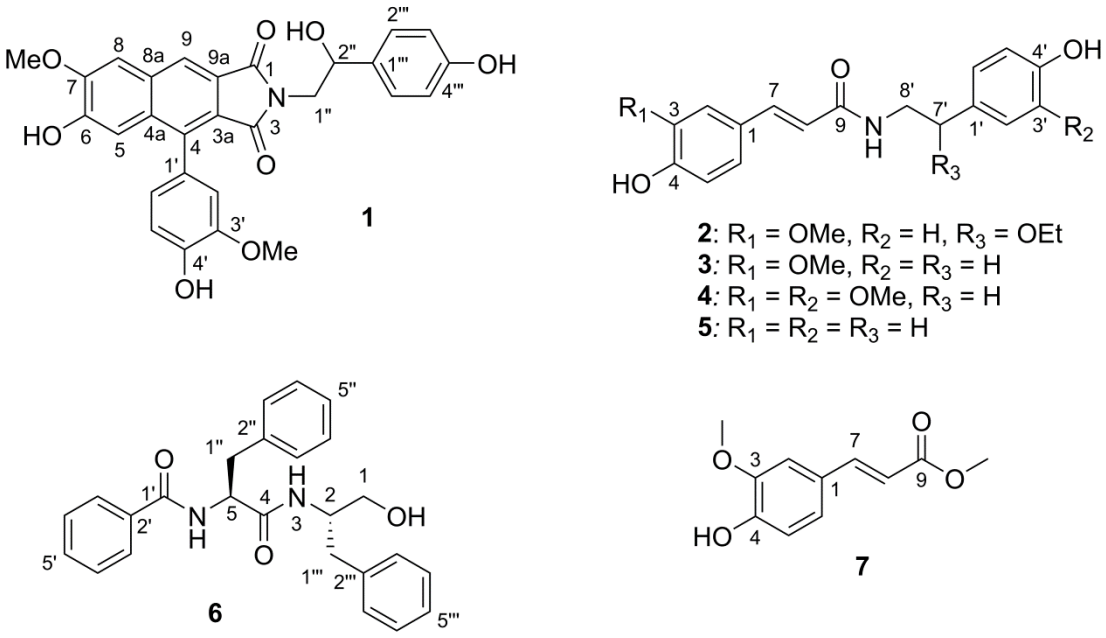

Figure 1. Chemical structures of compounds isolated from *P. oleracea*

2. Results and discussion

Compound **1** was a pale yellow powder, which showed a blue fluorescence spot at UV 365 nm, a black spot at UV 254 nm and remained orange when exposed to Dragendorff reagent on a TLC plate. UV (MeOH) λ_{max}: 284 nm (Figure S3). IR (KBr) ν_{max}: 3425, 1753, 1700, 1515, 1390, 1271, 1216 cm^{-1} (Figure S4). Its molecular formula $C_{28}H_{23}NO_8$ with 18 degrees of unsaturation was deduced from the ^{1}H-NMR, ^{13}C-NMR spectrum data (Table S1) and the HR-ESI^{-}-TOF-MS deprotonated molecular ionat *m/z* 500.1374 (calcd. 500.1351), along with the $[M-H_2O+H]^+$ ion at *m/z* 484.1397 in HR-ESI^{+}-TOF-MS (calcd. 484.1391) (Figure S5(a)(b)). At first sight, this compound appeared as a mixture of two very closely related molecules because some signals are doubled both in ^{1}H and in ^{13}C NMR spectra (Table S1-S2). We pursued with the structural determination considering each doubled peaks as one signal hoping that the structure would eventually enlighten us on the origin of the doubled signals.

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

3', C-4', C-6'; from H-6' to C-4, C-1', C-4', C-5', together with the ^{1}H-^{1}H COSY correlations from H-5' to H-6' suggesting that protons at δ 6.84/6.93, 6.75/6.78, and 6.95 defined a 1,3,4-trisubstituted phenyl moiety, which was linked to carbon C-4 at δ139.6. This phenyl ring bore a methoxy group in C-3' and a hydroxyl in C-4'.The position of the methoxy group could be inferred based on HMBC correlation H-5'/C-3' and was confirmed by the NOE correlation of the methyl with H-2'. The ^{1}H-^{1}H COSY spectrum showed the correlation H-2'''/H-6''', H-3'''/H-5''', and in HMBC the equivalent proton H-2'''/ H-6''' correlated to C-2", C-2'''/C-6''' and C-4'''; H-3'''/H-5''' to C-1''', C-3'''/C-5''', C-4'''. These are typical of a *para*-disubstituted phenyl ring. A hydroxyl could be placed in 4''' based on C-4''' chemical shift (δ158.2). The H-2'''/C-2" HMBC correlation indicated that C-2" was linked to C-1'''. The ^{1}H and ^{13}C chemical shifts in position 1" (δ_H3.67/3.70 and 3.88, δ_C46.1/46.2) are typical of a CH_2 linked to a nitrogen, while C-2" bore a hydroxyl (δ_H4.93, δ_C71.8/72.0). In HMBC, H-1" correlated to carbonyl groups C-1 and C-3 at δ170.2 and 169.1/169.3, respectively. In ^{1}H-NMR, only four signals remained to be attributed. These corresponded to a methyl 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), with respective carbons at δ_C123.30, 110.0, and 112.1. This group also contained nine quaternary carbons. These include C-4 at δ139.6 and carbonyls C-1 and C-3, which are linked to the already established peripheral groups. Protons H-8 and H-9 were not coupled to each other. Nevertheless, they correlated in NOE indicating that they were close to each other but not attached to a single phenyl ring. Also, H-8 correlated to C-9 and H-9 to C-8 in HMBC. Altogether, these indications led to position two *peri* protons on a naphthalene moiety. In HMBC, H-8 correlated to C-4a, C-6, C-7, and C-9. It was not possible to be sure of the position of the methoxyl and the hydroxyl based on HMBC only. However, strong NOE correlation between the methyl group and H-8 allowed to place the methoxyl substituent in C-7. H-9 correlated to C-8 and C-4a which further confirmed the relative place of H-8 and H-9. H-9 also correlated to C-1 and C-3a. It was therefore possible to link C-1 to C-9a. The C-3–C-3a bond could not be formally detected, but it was reasonable to link these carbons based on chemical shift of quaternary carbon C-3a (δ123.29). Since the last singlet proton H-5 at δ7.15 correlated to both C-6 and C-7, it had to be placed in *para*-position with respect to H-8. H-5 also correlated to C-8a and C-4. The latter established the position of the tetrasubstituted phenyl ring, which was confirmed by the NOE correlation between H-

5 and H-6′. C-9a could be detected neither in ^{13}C, nor in HMBC. NMR experiments were also conducted in DMSO-d_6. In this solvent, the ^{13}C NMR spectrum was of better quality and the only peak that did not correlate in HMBC was at δ125.20/125.23. This signal was attributed to C-9a. Compound 1 was determined to be the 6-hydroxy-2-(2-hydroxy-2-(4-hydroxyphenyl)ethyl)-4-(4-hydroxy-3-methoxyphenyl)-7-methoxy-1*H*-benzo[*f*]isoindole-1,3(2*H*)-dione, named oleraisoindole. The optical rotation of **1** was found to be -6° (0.1, MeOH). One center of atropoisomerism (the C-4–C-1′ bond) accounts for the duplication of many NMR signals. The relative proportion of the two rotamers as measured on the 3'-OMe proton signals is about 1:1.

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

From the MTT assay (Figure S32), compound **1** presented cytotoxicity at the concentration of 100 μM. The concentrations of 1 to 50 μM were therefore selected for the NO assay. Compound **1** significantly inhibited NO production at 20 μM on wards (Figure S33).

3. Experimental

3.1 General Experimental Procedures

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

Column chromatography (CC). TLC was conducted on silica gel GF_{254} (Qingdao Marine Chemical Co., Qingdao, China).

3.2 Plant materials

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

3.3 Extraction and isolation

The dried plants (150 kg) of *P. oleracea* L. were extracted with 10 fold of hot water twice times, each time for 2 h. The water extract was concentrated and then extracted with 1 fold of ethyl acetate three times. The ethyl acetate extract was concentrated under reduced pressure to obtain crude extract (200 g) and then subjected to chromatography on a silica-gel (200-300 mesh, about 1.5 kg) column (φ8 × 120 cm) with ethyl acetate and methanol as the gradient eluent (3:1, 1:1, 1:3, v/v) to obtain 20 fractions (400 mL each). The fractions of 1-2 were combined and repeatedly chromatographed by a 20-40 μm ODS-C_{18} (100 g) column (φ3 × 70 cm) with methanol and water as the gradient eluent (40:60, 60:40, 80:20, 100:0, v/v) to obtain 11 fractions (200 mL each). The fractions of 1-2 were separated by a Sephadex LH-20 (100 g) column (φ2 × 150 cm) with methanol-water (70:30, v/v) as the eluent to obtain 42 fractions (20 mL each). The fractions of 26-30 were purified with UHPLC and eluted with acetonitrile and water (30:70, v/v, 1.0 ml/min), affording oleraisoindole **(1)** (15 mg, purity > 98% with UHPLC), together with six known compounds, 7'-ethoxy-*trans*-feruloyltyramine **(2)** (20 mg, purity > 98% with UHPLC), *N-trans*-feruloyltyramine **(3)** (15 mg, purity > 98% with UHPLC), *N-trans*-feruloyl-3-methoxytyramine **(4)** (10 mg, purity > 98% with UHPLC), *N-trans-p*-coumaroytyramine **(5)** (8 mg, purity > 98% with UHPLC), aurantiamide **(6)** (20 mg, purity > 98% with UHPLC) and ferulic acid methyl ester **(7)** (30 mg, purity > 98% with UHPLC). Compound **1**-**6** turned orange when sprayed with Dragendorff reagent on thin-layer chromatography.

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

^{13}C NMR spectral data (in methanol-d_4 and DMSO-d_6), see Table S1. HR-ESI(+)-TOF-/MS m/z 484.1397 $[M\text{-}H_2O+H]^+$ (calcd: $C_{28}H_{22}NO_7$, 484.1391). HR-ESI(-)-TOF-/MS m/z 500.1374 $[M\text{-}H]^+$ (calcd: $C_{28}H_{22}NO_8$, 500.1351).

3.4 Biological assays

The RAW 264.7 macrophage cell was incubated in DMEM containing 10% heat inactivated fetal bovine serum and 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were seeded in 96-well plates at a density of 1×10^4 cells per well. Cell viability was performed using MTT assay. The cells were incubated with various concentrations (1, 10, 20, 50 and 100 μM) of oleraisoindole for 1 h, then 5 mg/ml MTT solution was added after stimulated with LPS (1 μg/mL) for 24 h and incubated for another 4 h at 37°C. The formazan was dissolved in 150 μl of DMSO. The absorbance was measured at 570 nm using a Bio-Tek microplate reader, cell viability was expressed as a percentage by comparing with the untreated cells. The production of NO was measured by Griess reaction. An equal volume of Griess reagent (0.1% naphthylethylethylene diamine dihydrochloride in H_2O and 1% sulphanilamide in 5% H_3PO_4) was added to the different concentrations of oleraisoindole cell culture supernatant (100 μl) collected before add MTT solution in MTT assay. The production of NO was detected at 550 nm by a Bio-Tek microplate reader, and was compared with sodium nutrite standard calibration curve.

4. Conclusion

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

Disclosure statement

No potential conflict of interest was reported by the authors.

Acknowledgements

We would like to thank to Professor Mingzhe Gao for HR-ESI-TOF-MS analysis in Dalian Institute of Chemical Physics.

Funding

This work was supported by the National Natural Science Foundation of China (Grant 81573546).

Supplementary material

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

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