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ABSTRACT

Syntheses of natural homoisoflavonoids, (±)-portulacanones A–C (**4**, **8** and **9**), portulacanone D (**6**), isolated from *Portulaca oleracea* L. (POL) and their derivatives (**3**, **5** and **7**) have been achieved for the first time along with the synthesis of known derivatives (**1** and **2**) and their *in vitro* inhibitory effect against NO production in LPS-induced RAW-264.7 macrophages was evaluated as an indicator of anti-inflammatory activity. All the compounds tested had a concentration-dependent inhibitory effect on NO production by RAW-264.7 macrophages without obvious cytotoxicity. Compounds **3** (97.2% at 10 μ M; IC₅₀ = 1.26 μ M) followed by **6** (portulacanone D) (92.5% at 10 μ M; IC₅₀ = 2.09 μ M), **1** (91.4% at 10 μ M; IC₅₀ = 1.75 μ M) and **7** (83.0% at 10 μ M; IC₅₀ = 2.91 μ M) were the most potent from the series. This finding was further correlated with the suppressed expression of iNOS induced by LPS. Our promising preliminary results may provide the basis for the assessment of compound **3** as lead structure for a NO production-targeted anti-inflammatory drug development and also could support the usefulness of POL as a folklore medicinal plant in the treatment of inflammatory diseases.

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Inflammation is a fundamental protective response of cells/tissues of the body to infection by pathogens, damaged cells or irritants.^{1,2} It is regarded as the beginning of a healing process and can be classified as either acute or chronic. The one is an adaptive host defense mechanism against infection or injury and is self-limiting whereas the other may lead to various ailments including diabetes, arthritis and cancer. During the inflammatory process, various chemical mediators such as nitric oxide (NO), prostaglandins (PG), vasoactive amines (histamine, serotonin), leukotrienes (LT), and cytokines (tumor necrosis factor and interleukins-1, 12) are released as plasma proteins, or that come from cells like mast cells, neutrophils, platelets and monocytes/macrophages. These mediators bind to specific target receptors on the cells and may increase vascular permeability, stimulate smooth muscle contraction, promote neutrophil chemotaxis, increase direct enzymatic activity, induce pain and/or mediate oxidative damage.³

NO, synthesized from L-arginine by a family of enzymes- the nitric oxide synthases (endothelial-NOS, neuronal-NOS and inducible-NOS), is a diffusible, small and transient free-radical molecule having a multifaceted role in human physiology and pathophysiology.⁴ The concentration of NO plays a decisive role in the patho-

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https://doi.org/10.1016/j.bmcl.2018.04.037 0960-894X/© 2018 Elsevier Ltd. All rights reserved. genesis of inflammation. Physiologically vital amounts of NO produced by endothelial- and neuronal-NOS promote cell survival and proliferation, whereas higher levels of NO from inducible-NOS favor cell cycle arrest and apoptosis.⁵ Interaction of reactive oxygen species (ROS) like superoxide and hydroxyl radicals with NO leads to reduction in NO concentration, antagonizes its signaling activity and the resulting reactive nitrogen species (RNS) can also increase oxidative and nitrosative stress responses.⁶ Although non-steroidal anti-inflammatory drugs (NSAIDs), steroids and anti-histamines are commonly preferred for the treatment of pain, inflammation and fever, they are not without their shortcomings. Thus, pharmacological interference with the NO production cascade is claimed as a promising strategy among many of the therapeutic intervention in inflammatory diseases.

Naturally occurring flavonoid compounds, specifically the subclass of flavonoids known as homoisoflavonoids, have displayed great biological potential in recent years.^{7,8} Previous studies have shown that homoisoflavonoids exhibit antioxidant, anti-inflammatory, antibacterial, antifungal, antiviral, cytotoxic, antimutagenic, and antidiabetic activities.^{8,9} Besides these, a few derivatives displayed enzyme inhibitory properties.¹⁰

The Tan group isolated four homoisoflavonoids named portulacanones A–D (Fig. 1) from the plant *Portulaca oleracea* L. (POL), a widespread medicinal plant that is used not only as an edible plant, but also as a folk medicine for alleviating a wide spectrum of

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Fig. 1. Structures of portulacanones A–D (4, 8, 9 and 6) and their derivatives (1–3, 5 and 7).

diseases.¹¹ Upon isolation these compounds were evaluated and showed *in vitro* cytotoxic activities towards four human cancer cell lines, including SGC-7901, NCI-H460, K-562 and SF-268.¹¹ Intriguing bioactivity of POL, especially promising anti-inflammatory activity,¹² and no report on the synthesis of portulacanones A–D led us to attempt the synthesis and study their NO inhibitory activity. As part of our ongoing interest^{13,14} in the synthesis of bioactive natural products and their analogues as potent NO production inhibitors, herein, we report the synthesis and *in vitro* study of (±)-portulacanones A–C (**4**, **8**, and **9**) portulacanone D (**6**) and their derivatives (**1–3**, **5** and **7**) (Fig. 1).

The synthesis of homoisoflavonoids (1-9) commenced with the Friedel-Crafts acylation of commercially available 1,3,5trimethoxybenzene (10) (Scheme 1). Treatment of 10 with acetic anhydride in the presence of boron trifluoride diethyl etherate (BF₃·Et₂O) provided compound 11 in 89% yield. Compound 11 underwent selective ortho-demethylation using AlCl₃ to furnish compound **12** in 87% yield. Another acetophenone **14** was accessed from compound **13**. Acetylation of compound **13** and subsequent Fries rearrangement afforded 14 in an excellent yield of 93% over two steps. Protection of salicylaldehyde (15) as its ethoxymethyl (EOM) ether (16) using chloromethyl ethyl ether (EOM-Cl), K₂CO₃ and catalytic tetrabutylammonium iodide (TBAI) proceeded in 83% yield. Next, condensation of commercially available 2hydroxy-4-methoxyacetophenone (17), compounds 12 and 14 with N,N-dimethylformamide dimethyl acetal (DMF-DMA) followed by acid treatment of the resulting enamino ketones gave the corresponding 4H-chromen-4-ones 18a-18c in 88-91% yields, respectively. Catalytic hydrogenation of 18a-18c delivered the corresponding chroman-4-ones 19a-19c in 83-87%, respectively.

Having the key fragments 19a-19c and 16 in hand, next, we set out to explore the key Aldol condensation reaction. At first, we tried the reactions between **19b** and **15** using *p*-toluenesulfonic acid (pTsOH) in benzene which was not successful. Next, condensation of **19b** and **16** using piperidine as a base in benzene as well as in DMF at 30-100 °C resulted in low yield (<20%) of product (20b) formation. Gratifyingly, we identified KOH in EtOH/H₂O (5/1) was effective for the condensation of 19b and 16. Subsequently, the reaction of 19a and 19c with 16 provided 20a (48%) and 20c (25%), respectively. Deprotection of 20a-20c using 1 N HCl gave the compounds 1, 3 and 7 in high yields. Catalytic hydrogenation of 1, 3 and 7 afforded the homoisoflavonoids 2, 4 and 8 in 86–91% yields, respectively. Finally, selective ortho-demethylation of **4**, **8** and **3** using 1.0 M BCl₃ (in CH₂Cl₂) smoothly furnished the leftover three target compounds 5, 9 and 6, respectively. All the final products (1-9) structures were settled from their spectral data (¹H and ¹³C NMR and MS) (see the Supplementary information).

NO inhibitory potential of the synthesized homoisoflavonoids (1–9) was monitored *in vitro* by incubating RAW 264.7 macrophage cell lines with bacterial lipopolysaccharides (LPS), a major structural component of the outer wall of Gram-negative bacteria. It is well known that treatment of RAW 264.7 macrophages with LPS induces NO production. N^{G} -Monomethyl-L-arginine (L-NMMA) has been reported to significantly suppress NO production.¹⁵ In this study, RAW 264.7 macrophages were treated with LPS and 0.1, 1, 10, 25, 50 and 100 μ M concentrations of compounds **1–9** and L-NMMA as control, then NO production and cell viability were measured. However, at 25, 50 and 100 μ M concentrations, compounds **1–9** exhibited almost same level activity (Fig. 2). Significant NO suppression changes were observed at 1–10 μ M. Hence, we discussed the activities at 1 and 10 μ M concentrations only. The magnitude of NO released from macrophages was determined by measuring the concentration of nitrite, a stable oxidized product of NO, in the culture supernatant using the Griess reagent.

All the compounds tested (1–9) had a concentration-dependent inhibitory effect NO production by 264.7 macrophages (Table 1). The percentage of NO inhibition ranged from 97.2% to 15.9% at the highest (10 μ M) concentrations. Of the 9 compounds (1–9), 4 compounds i.e., **3** (97.2%), followed by **6** (portulacanone D) (92.5%), **1** (91.4%) and **7** (83.0%) showed the strongest inhibitory activities at 10 μ M (Table 1 and Fig. 2). At the lowest concentration (1 μ M), compound **3** still significantly reduced the NO production (38.5%) by 264.7 macrophages. IC₅₀ values of **1–9** were evaluated by GraphPad Prism 4.0 software and showed 1.75, 14.17, 1.26, 14.17, 12.42, 2.09, 2.91, 13.43 and 12.16 μ M respectively (Table 1).

The cytotoxicity of the compounds against RAW 264.7 macrophages was also tested by MTT assay to ascertain that the observed NO inhibitory effect of 1-9 was not due to the cell death. None of the tested compounds exerted detectable cytotoxicity at 10 µM concentration for 24 h, which was leading to effective inhibition of NO production (Table 2). Although, the compounds 1-9 had good activity against NO production at 50 μ M and 100 μ M, this is most likely due to cytotoxic effects of 1-9 on the macrophages (Fig. 2 and Table 2). Percentages of cell viability at 50 μ M and 100 µM respectively were only in the range of 10.7-58.7% and 10.9-44.0%. We further evaluated whether these inhibitory effects are related to iNOS modulation using Western blot analysis. As shown in Fig. 3, the results were in accordance with the findings related to NO production (Table 1 and Fig. 2), the protein expression of iNOS induced by LPS in RAW 264.7 cells was markedly inhibited by compounds 1, 3, 6 and 7 treatment. However, these compounds did not affect the expression of β -actin, the housekeeping gene. This indicates that the reduced expression of iNOS due to these compounds exposure was responsible for the inhibition of NO production. As we screened a limited number of compounds i.e., only 9 compounds, thorough structure-activity relationship (SAR) analysis is not possible. However, the following might be worth noting: 1) double bond between C3 and C11 appears to be crucial for the observed inhibition of NO as compounds 1, 3, 6 and **7** were the most efficacious. 2) Within the C3 and C11 double bond containing compounds (1, 3, 6 and 7), compounds with two

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Scheme 1. Reagents and conditions: (a) Ac₂O, BF₃·Et₂O, EtOAc, rt, 4 h, 89%; (b) AlCl₃, CH₂Cl₂, reflux, 22 h, 87%; (c) (i) Ac₂O, Et₃N, CH₂Cl₂, rt, 6 h; (ii) BF₃·Et₂O, AcOH, 70 °C, 2 h, 93% (over 2 steps); (d) chloromethyl ethyl ether, K₂CO₃, tetrabutylammonium iodide, acetone, 0 °C–rt, 24 h, 83%; (e) *N*,*N*-dimethylformamide dimethyl acetal, benzene, 90 °C, overnight, 88–91%; (f) H₂, Pd/C (10%), MeOH, rt, 0.75–1 h, 83–87%; (g) KOH, EtOH/H₂O (5/1), 25–30 °C, 10–24 h, 25–48%; (h) 1 N HCl, MeOH, 55 °C, 1 h, 85–88%; (i) H₂, Pd/C (10%), MeOH, rt, 0.75–1 h, 86–91%; (j) 1.0 M BCl₃ (in CH₂Cl₂), CH₂Cl₂, 0 °C–rt, 3 h, 83–86%.



Fig. 2. Inhibitory activity of homoisoflavonoids 1–9 on NO production in LPSactivated RAW 264.7 macrophages.

Table 1NO inhibitory effects of homoisoflavonoids (1-9).

Compound	NO Production (% inhibition) ^{a,b}			
	1 μM	10 µM	IC ₅₀ μM	
LPS 1 2 3 4 5 6 7	$100.0 \pm 0.8 (0.0)$ $71.6 \pm 1.4 (28.4)^{**}$ $90.8 \pm 1.7 (9.2)^{*}$ $61.5 \pm 1.7 (38.5)^{**}$ $102.8 \pm 1.7 (-2.8)$ $97.2 \pm 1.7 (2.8)$ $78.9 \pm 2.0 (21.1)^{**}$ $83.8 \pm 1.9 (16.2)^{**}$	100.0 ± 0.8 (0.0) 8.6 ± 1.2 (91.4) 80.3 ± 6.5 (19.7) 2.8 ± 0.7 (97.2) 79.5 ± 7.3 (20.5) 62.0 ± 2.1 (38.0) 7.5 ± 0.8 (92.5) 17.0 ± 0.8 (83.0)	1.75 14.17 1.26 14.17 12.42 2.09 2.91	
8 9 1-NMMA	124.6 ± 17.6 (-24.6) 111.8 ± 1.3 (-11.8) [*] 112.5 ± 4.6 (-12.5)	84.1 ± 2.7 (15.9) 65.9 ± 2.9 (34.1)*** 85.8 ± 6.4 (14.2)	13.43 12.16 16.11	

^a The results are reported as mean value ± SEM for n = 3. Statistical significance is based on the difference when compared with LPS-treated groups ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$).

^b % Inhibition is based on LPS as shown in parenthesis.

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Table 2	
Proliferation effect of homoisoflavonoids 1–9.	

Compound	Proliferation effect ^a				
	1 μM	10 µM	50 μM	100 µM	
Medium (MED)	100.0 ± 2.4	100.0 ± 2.4	100.0 ± 2.4	100.0 ± 2.4	
1	101.8 ± 1.5	97.7 ± 13.3	$11.0 \pm 0.1^{***}$	$11.1 \pm 0.2^{***}$	
2	$152.1 \pm 2.4^{***}$	118.3 ± 1.1	$49.4 \pm 3.9^{***}$	$31.5 \pm 0.3^{***}$	
3	107.6 ± 12.0	113.6 ± 5.5	$13.3 \pm 1.6^{***}$	$11.7 \pm 0.9^{***}$	
4	98.6 ± 10.5	164.6 ± 8.3**	$55.2 \pm 1.0^{***}$	$41.3 \pm 1.4^{***}$	
5	97.7 ± 0.9	81.1 ± 4.8**	$34.2 \pm 2.0^{***}$	$12.5 \pm 0.2^{***}$	
6	94.0 ± 4.4	97.0 ± 4.6	$10.7 \pm 0.1^{***}$	$11.0 \pm 0.1^{***}$	
7	94.6 ± 3.5	93.4 ± 2.8	$11.8 \pm 0.1^{***}$	$10.9 \pm 0.3^{***}$	
8	89.6 ± 2.9	$87.2 \pm 3.2^{*}$	$58.7 \pm 2.6^{***}$	$44.0 \pm 1.4^{***}$	
9	97.6 ± 5.4	96.7 ± 2.0	$41.1 \pm 5.3^{***}$	$15.5 \pm 0.3^{***}$	

^a The results are reported as mean value \pm SEM for n = 3 ($^{\circ}P < 0.05$, $^{\circ\circ}P < 0.01$ and $^{\circ\circ\circ}P < 0.001$).



Fig. 3. Effects of compounds 1-9 on iNOS expression (Western blot).

methoxy (-OMe) (compound **3**) or one methoxy (-OMe) and one hydroxy (-OH) group (compound **6**) containing compounds had better NO inhibitory activity than the compounds with one methoxy (-OMe) group (compound **1**) and three methoxy (-OMe) groups (compound **7**). 3) Strong NO inhibitory effect of **6** (portulacanone D) could help to support the usefulness of POL plant in the treatment of inflammatory diseases and to validate the traditional indication of this species.

In conclusion, we have demonstrated the first synthesis of natural homoisoflavonoids, (±)-portulacanones A-C (4, 8 and 9), portulacanone D (6) and their derivatives (3, 5 and 7) along with the synthesis of known derivatives (1 and 2) from commercially available building blocks. Later, in vitro inhibitory effect of these compounds against NO production in LPS-treated RAW-264.7 macrophages was evaluated as an indicator of anti-inflammatory activity. All the homoisoflavonoids tested had a concentrationdependent inhibitory effect on NO production by RAW-264.7 macrophages without marked cytotoxicity. Compounds 3 (97.2% at 10 μ M; IC₅₀ = 1.26 μ M) followed by **6** (portulacanone D) (92.5% at 10 $\mu M;$ IC_{50} = 2.09 $\mu M),$ 1 (91.4% at 10 $\mu M;$ IC_{50} = 1.75 $\mu M)$ and **7** (83.0% at 10 μ M; IC₅₀ = 2.91 μ M) were the most potent from the series. This finding was additionally correlated with the suppressed expression of iNOS induced by LPS. The information gathered from this promising preliminary study may provide the basis to consider the compound **3** as a lead structure for a NO production-targeted anti-inflammatory drug development and also contribute further scientific evidence about the usefulness of POL as a folklore medicinal plant in the treatment of inflammatory diseases.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.bmcl.2018.04.037.

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