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Synthesis and *in vitro* evaluation of homoisoflavonoids as potent inhibitors of nitric oxide production in RAW-264.7 cells

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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 μM; IC_{50} = 1.26 μM) followed by **6** (portulacanone D) (92.5% at 10 μM; IC_{50} = 2.09 μM), **1** (91.4% at 10 μM; IC_{50} = 1.75 μM) and **7** (83.0% at 10 μM; IC_{50} = 2.91 μM) 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 a 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.

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 pathogenesis 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 antihistamines 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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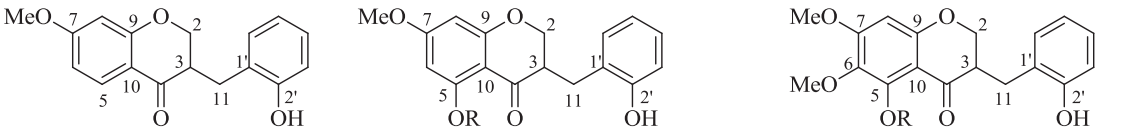

1 $\Delta^{3,11}$

2

3 R = Me, $\Delta^{3,11}$

4 R = Me (± portulacanone A)

5 R = H

6 R = H, $\Delta^{3,11}$ (portulacanone D)

7 R = Me, $\Delta^{3,11}$

8 R = Me (± portulacanone B)

9 R = H, (± portulacanone C)

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 1,3,5-trimethoxybenzene (**10**) (Scheme 1). Treatment of **10** with acetic anhydride in the presence of boron trifluoride diethyl etherate ($BF_3{\cdot}Et_2O$) provided compound **11** in 89% yield. Compound **11** underwent selective *ortho*-demethylation using $AlCl_3$ 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), K_2CO_3 and catalytic tetrabutylammonium iodide (TBAI) proceeded in 83% yield. Next, condensation of commercially available 2-hydroxy-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 4*H*-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 (*p*TsOH) 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 $EtOH/H_2O$ (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 BCl_3 (in CH_2Cl_2) 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 ^{13}C 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 μM concentrations of compounds **1**–**9** and L-NMMA as control, then NO production and cell viability were measured. However, at 25, 50 and 100 μM concentrations, compounds **1**–**9** exhibited almost same level activity (Fig. 2). Significant NO suppression changes were observed at 1–10 μM. Hence, we discussed the activities at 1 and 10 μM 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 μM) 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 μM (Table 1 and Fig. 2). At the lowest concentration (1 μM), compound **3** still significantly reduced the NO production (38.5%) by 264.7 macrophages. IC_{50} 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 μM 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 μM and 100 μM, 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 μM 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