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Original article

Synthesis and biological evaluations of novel apocynin analogues

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ABSTRACT

We have designed and synthesized a series of novel apocynin analogues, and evaluated their biological activity. Compound **10**, an apocynin dimer analogue, compound **12**, the lipoic acid (LA) and apocynin conjugate, were the most potent in protecting cells from *lipopolysaccharide* (LPS)-induced cytotoxicity, had significant activity scavenging ROS induced by LPS, and greatly decreased LPS-induced $P67^{phox}$ protein expression. SAR analysis suggests that modification of apocynin can increase its activity. Our results demonstrate that arming apocynin with a powerful antioxidant such as lipoic acid is a valid strategy to design new apocynin analogues with enhanced biological activity.

1. Introduction

Apocynin (4-hydroxy-3-methoxyacetophenone, Fig. 1), a methoxycatechol first isolated from the roots of *Apocynum cannabinum* (Canadian hemp) in 1883, was originally used as remedies for dropsy and heart illness [1]. Recent investigations have found that apocynin is an efficient inhibitor of NADPH oxidase [2–5]. Apocynin has been investigated as a therapeutic candidate for inflammation-mediated diseases, including jaundice, asthma [6,7] and cardiovascular diseases [8].

Although apocynin has been widely used as an inhibitor of NADPH oxidase; the exact mechanism by which it inhibits NADPH oxidase is still not well understood. Apocynin was found to be metabolized into its corresponding dimer [9], trimer [10] or even oligomer, which were considered to be the actual active component inhibiting NADPH oxidase by disrupting the interaction of $p47^{phox}$ with $p22^{phox}$ [7,9,10]. However, Heumueller *et al.* proposed a different mechanism that apocynin is not an inhibitor of vascular NADPH oxidase, but an antioxidant [11].

The biological effects and mechanism of action of apocynin are extensively investigated; however, few reports are seen regarding to its structure–activity relationship (SAR). To uncover its SAR, better understand mechanism(s) of action and find more potent NADPH oxidase inhibitors, we have designed and synthesized new apocynin analogues (Fig. 2) and investigated their preliminary biological activities in vitro and in cell cultures.

In this work, we first modified apocynin's 5-position to synthesize apocynin amides and amines, *i.e.*, compounds **3**, **6** and **8**. Since apocynin was converted to its dimer, we then synthesized an apocynin dimer, *i.e.*, compound **10** to help understand its mechanism of action.

In order to increase antioxidative activity, we coupled two of the most powerful antioxidants, *i.e.*, a nitrone and α-lipoic acid (LA) to apocynin to make compounds **11** and **12**. Nitrones have been widely used as spin traps in chemistry. In the last two decades, the therapeutic effects of nitrones against atherosclerosis, septicemia, stroke, and Alzheimer's disease have been intensively investigated [12,13]. Organic molecules incorporating a nitrone moiety act as reactive oxygen species (ROS) scavenger against oxidative challenges. LA is biogenic and one of the most powerful antioxidants known. LA directly terminates free radicals, chelates transition metal ions (iron and copper), and increases cytosolic glutathione and vitamin C levels [14,15]. These diverse actions suggest that LA acts by multiple mechanisms both physiologically and pharmacologically [16]. LA has been successfully employed in the treatment of diabetic polyneuropathy for decades [14,17].

In order to evaluate the biological activity of the apocynin analogues, we first investigated their protective activity against

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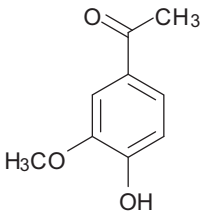


Fig. 1. Structure of apocynin.

LPS-induced cytotoxicity by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. We then investigated the ROS-scavenging activity of compounds **10**, **11** and **12** in RAW 264.7 cells by a fluorescence-based assay. Finally, we determined their inhibitory activity against LPS-induced $P67^{phox}$ expression. We herein report our experimental results.

2. Chemistry

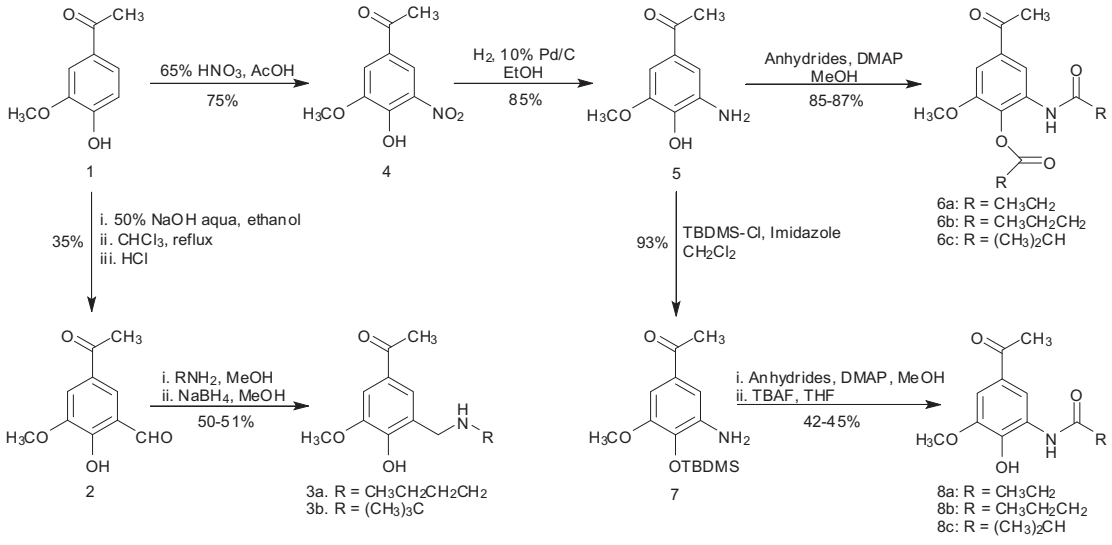
We first synthesized apocynin amides and amines, **3a**, **3b**, **6a**–**6c** and **8a**–**c** (Scheme 1), to find their effects on biological activity. Briefly, compound **2** was prepared by the Reimer–Tiemann reaction. Butylamine and tert-butylamine were then conjugated with compound **2**, respectively, to afford the corresponding imines, which were reduced immediately to produce the more stable amines **3a** and **3b**. Compound **1** was nitrated to give a nitro **4**. Catalytic hydrogenation of the latter over 10% Pd/C in absolute ethanol afforded amine **5**. Direct condensation of **5** with corresponding carboxylic acids produced compounds **6a**–**c**. Protection of the hydroxyl group of **5** by TBDMS–Cl in dichloromethane gave compound **7**. Amidation of **7** with various acid anhydrides in THF catalyzed by DMAP yielded the corresponding amides, which were then treated with TBAF in THF removing TBDMS to produce compounds **8a**–**c**.

Next, we synthesized an apocynin dimer **10**. The latter was structurally homologous to diapocynin, reported by Johnson *et al.* to be the real active component in peroxidization process *in vivo*. The synthesis of compound **10** is shown in Scheme 2. Treatment of compound **2** with **5** produced Schiff base **9**, which was reduced using sodium borohydride afforded the target compound **10**.

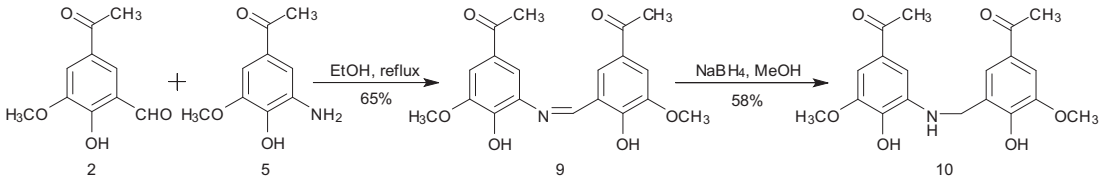
We then synthesized compounds **11** and **12**. Both of them are armed with a powerful antioxidative moiety, *i.e.,* nitrone or LA. The synthesis of **11** is shown in Scheme 3. Compound **2** was reacted with N-tert-butylhydroxylamine, prepared on the day of use by reduction of 2-methyl-2-nitropropane catalyzed by activated zinc, to afford **11**.

The synthesis of **12** is shown in Scheme 4. Amidation of compound **7** with LA in THF catalyzed by EDCI and HOBT followed by treatment with TBAF in THF afforded target **12**.

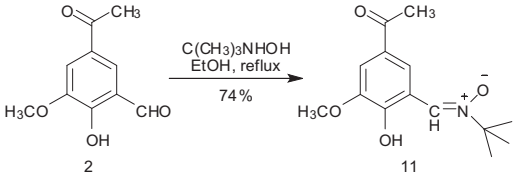
Fig. 2. Structure of apocynin amides and amines.



Scheme 1. Synthesis of apocynin amides and amines.



Scheme 2. Synthesis of compound **10**.



Scheme 3. Synthesis of compound **11**.

3. Biological evaluations and discussion

3.1. Protective effects against LPS-induced cytotoxicity

To determine the new compounds' biological activities, we first investigated their protective effect against LPS-induced cytotoxicity by the MTT assay in RAW 264.7 macrophage cells by the procedure described by Kim and Ha [18]. RAW 264.7 cells were cultured and treated with various drugs for 1 h prior to addition of LPS. After 24 h of incubation, cell viability was determined. The results are shown in Fig. 3. Apocynin (Apo) had minimum protective effects against LPS-induced cytotoxicity in this assay, and the result was consistent with what had been reported in the literature [19–21]. The reason may be that it needs to be converted into its active metabolites after a peroxidation process *in vivo* [9].

The LA substituted analogue **12** is the most potent compound in this assay. This finding confirmed that an antioxidant moiety could ameliorate the LPS-induced cell damage (Fig. 3). The apocynin dimer **10** also had strong protective effect against the LPS-induced cell damage. Interestingly, the structure of compound **10** is analogous to diapocynin (5,5′-dehydrodiacetovanillone), which was synthesized by oxidative coupling of apocynin [22]. Diapocynin is a metabolite of apocynin, which has anti-inflammatory and anti-oxidative properties. In LPS-stimulated PBMC cells diapocynin was more effective than apocynin [23]. Further studies are needed to confirm if apocynin dimer is the active form. These findings indicate that compounds **10** and **12** may increase apocynin's protective effect in this assay.

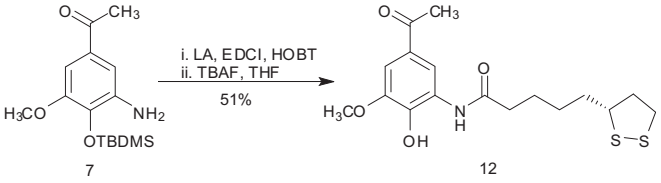
3.2. Intracellular ROS-scavenging activity

Next, we investigated the ROS-scavenging activity of compounds **10**–**12** in RAW 264.7 cells by a fluorescence-based assay (Fig. 4). RAW 264.7 cells were labeled with the fluorescence probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA). DCFH-DA enters cells and its DCFH moiety interacts with ROS to emit fluorescence [24].

In the absence of any drug, cells treated with LPS showed strong presence of ROS. At the tested concentrations (0.1, 1 and 10 μM), apocynin (Apo) and compound **11** had minimum activity scavenging LPS-induced ROS. Compounds **10** and **12** dose-dependently reduced ROS level. Recent studies have shown that both α-lipoic acid and apocynin can reduce intracellular reactive oxygen species [25,26]. Diapocynin can inhibit inflammatory factors such as TNF-α, IL-10, and gp91^{phox} mRNA expression in LPS-induced PBMC cells. Compound **10** is analogous to diapocynin. It will be interesting to find out if compound **10** and diapocynin work by the same mechanisms.

3.3. Inhibition of LPS-induced P67^{phox} protein expression

Macrophage RAW 264.7 cells were pre-treated with 0.1–10 μM apocynin (Apo), compounds **10**–**12** for 1 h, and were then stimulated with LPS (1 μg/ml) for 24 h. Expression of P67^{phox} protein was detected by Western blot analysis (Fig. 5). As is shown in Fig. 5, LPS induced considerable expression of P67^{phox} proteins. Apo and its nitrone analogue **11** did not reduce P67^{phox} protein expression at concentrations up to 10 μM. Others reported that Apo at 100 μM



Scheme 4. Synthesis of compound **12**.

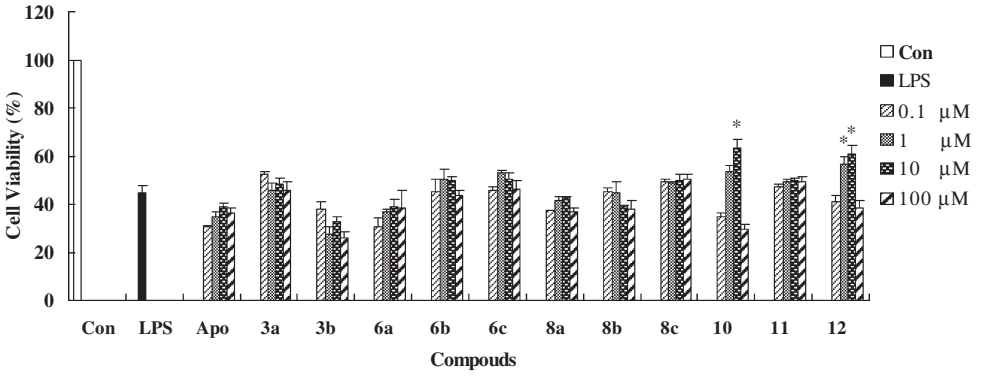


Fig. 3. Protective effects of Apo and its analogues against LPS-induced cytotoxicity in RAW 264.7 cells.

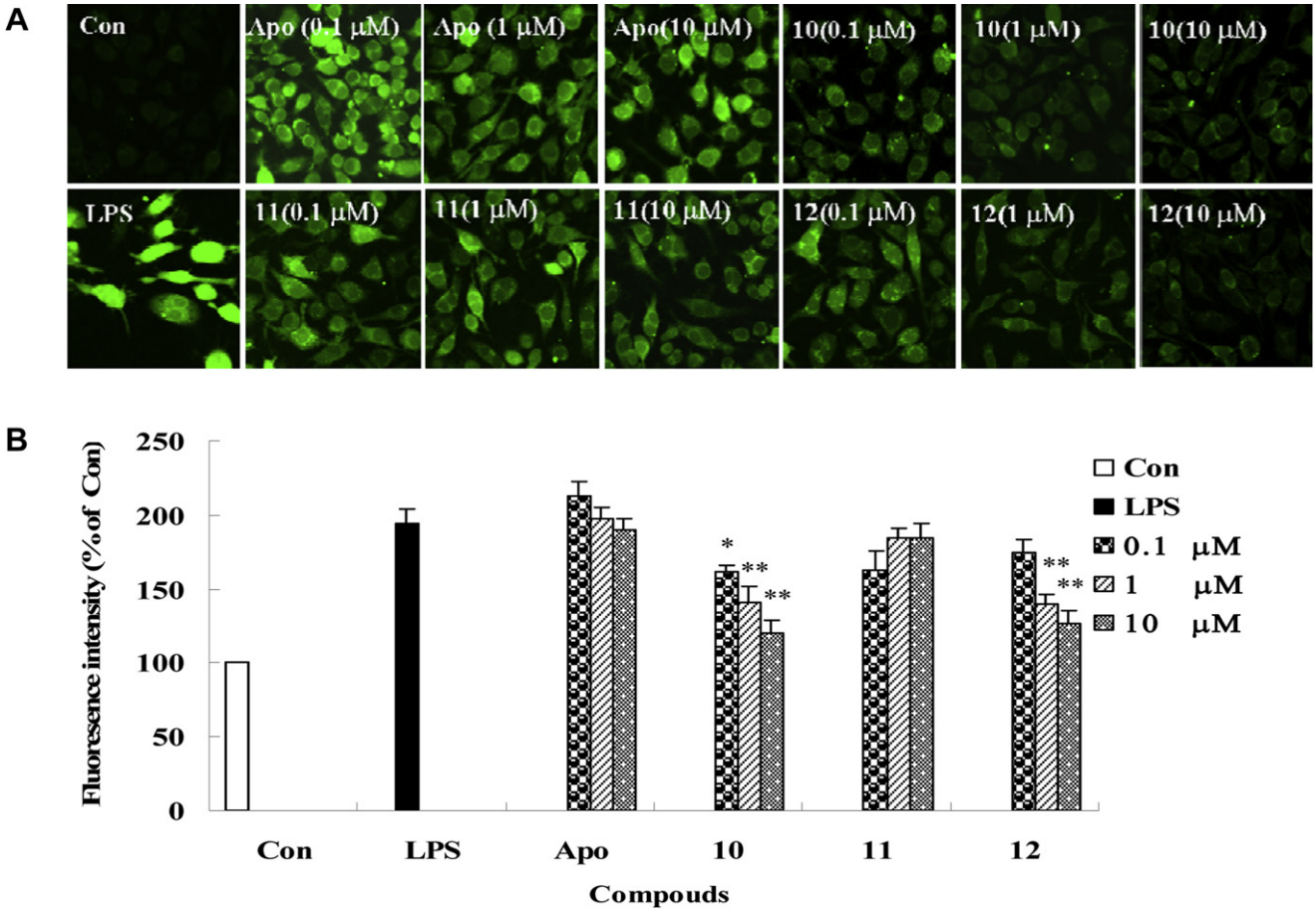


Fig. 4. ROS-scavenging effect of Apo and compounds **10**–**12** in RAW 264.7 cells.

effectively decreased P67^{phox} expression in leptin-induced cardiomyocytes and completely suppressed NADPH oxidase at 1 mM [27–29]. Both the dimer **10** and the LA analogue **12** significantly inhibited expression of P67^{phox} protein dose-dependently from 0.1 to 10 μM. Bhatti *et al.* reported that kidney expression of NADPH oxidase subunits in diabetic rats can be reduced by α-lipoic acid [30]. Kanegae *et al.* reported that diapocynin inhibited NADPH oxidase more effectively than apocynin through gp91^{phox} mRNA expression [22]. The results demonstrated that compounds **10** and **12** are effective NADPH oxidase inhibitors.

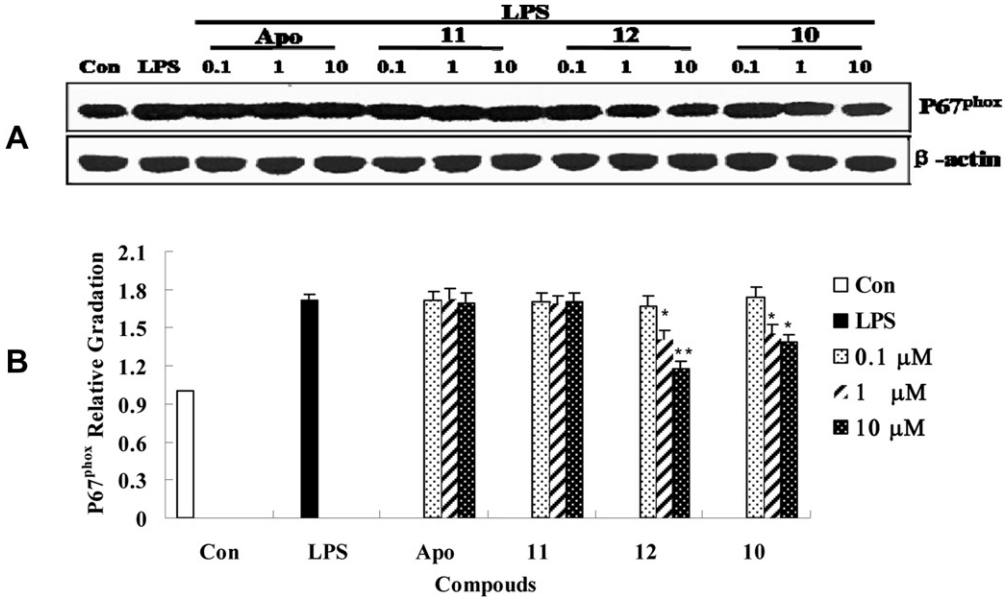


Fig. 5. Inhibition effect of Apo and compounds **10**–**12** against LPS-induced P67^{phox} protein expression.

4. Conclusions

In this study, we have synthesized a series of novel apocynin analogues. Their biological activity was first evaluated by the MTT assay against LPS-induced cytotoxicity. Among the new compounds, the dimer **10** and the LA-substituent **12** were the most potent protecting cells from LPS-induced cytotoxicity. Apocynin was found to have minimum activity. Compounds **10** and **12** significantly reduced LPS-induced ROS production at a concentration as low as 0.1 μM in RAW 264.7 cells. Compounds **10** and **12** greatly inhibited LPS-induced $P67^{phox}$ protein expression. These results seem to support the notion that apocynin needs to be converted to a dimer to be active [9,11]. Our results demonstrate that coupling a powerful antioxidant moiety such as LA to apocynin is a valid strategy for improving apocynin's biological activity.

5. Experimental protocols

5.1. General methods

All chemicals (reagent grade) used were commercially available. Melting points were measured using a Mel-Temp (X_7L_{20}, Beijing), and are uncorrected. NMR spectra were recorded at ambient temperature on a 400 MHz spectrometer (AV-400, Bruker) in $CDCl_3$, CD_3OD or DMSO-d_6. Electrospray ionization mass spectra (ESI-MS) were obtained in the positive ion detection mode on a Finnigan LCQ Advantage MAX mass spectrometer (Applied Biosystems, 4000 Q TRAP). Elemental analysis was performed at the Jinan University Experimental Center.

5.2. 5-Acetyl-2-hydroxy-3-methoxybenzaldehyde (**2**)

To apocynin **1** (5 g, 30.1 mmol) and triethylamine (3 ml) in ethanol (50 ml), was added a solution of sodium hydroxide (25 g) in water rapidly. The resulting solution was heated to 70 °C on a steam bath. Chloroform (39 g, 300 mmol) was added dropwise. Stirring was continued for 2 h after all the chloroform had been added. After cooling to room temperature, the reaction mixture was acidified by hydrochloric acid to pH = 1. The chloroform phase was collected, and the aqueous phase was extracted by dichloromethane (20 ml × 3). The mixture of organic phase was washed with water (10 ml × 3) and was then dried using anhydrous sodium sulfate. Solvent was removed *in vacuo* and the product was purified by column chromatography, eluting with ethyl acetate/petroleum ether (2:3, v/v), to afford 2.2 g (35% yield) of **2** as a yellow powder, mp: 156–157 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): 11.47 (s, 1H, OH), 10.00 (s, 1H, CHO), 7.83 (d, 1H, J = 1.6 Hz, ArH), 7.74–7.73 (d, 1H, J = 1.6 Hz, ArH), 3.98 (s, 3H, OCH_3), 2.62 (s, 3H, CH_3CO). MS (ESI) m/z 193 $[M-H]^+$, 195 $[M+H]^+$. Analysis calculated for $C_{10}H_{10}O_4 \cdot 0.67H_2O$: C, 58.25; H, 5.54. Found: C, 58.04; H, 5.78.

5.3. General procedure for synthesis of compounds ***3a*** and ***3b***

Compound **2** (200 mg, 1.03 mmol) was first dissolved in methanol (20 ml). A solution of alkylamine in methanol (5 ml) was added dropwise and the reaction mixture was stirred at room temperature for 1 h to afford a Schiff base intermediate. The reaction mixture was then cooled to 0 °C under an ice bath, and sodium borohydride (13 mg, 0.34 mmol) was added in single portions with vigorously stirred. Ice bath was removed and the reaction mixture was stirred at room temperature for 2 h. Ice water (10 ml) was added to dissolve the excess sodium borohydride. The reaction mixture was extracted with dichloromethane (10 ml × 3) and the organic layer was dried using anhydrous sodium sulfate. Solvent was removed *in vacuo* to afford **3a** and **3b**.

5.3.1. 1-(3-((Butylamino)methyl)-4-hydroxy-5-methoxyphenyl) ethanone (***3a***)

Synthesized from **2** (200 mg, 1.03 mmol) and butylamine (150 mg, 2.06 mmol). Brown solid (131 mg, 51% yield), mp: 161–163 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): 7.44 (d, 1H, J = 1.6 Hz, ArH), 7.29 (d, 1H, J = 1.6 Hz, ArH), 4.07 (s, 2H, $ArCH_2N$), 3.93 (s, 3H, OCH_3), 2.70–2.68 (t, 2H, J = 7.1 Hz, NCH_2), 2.53 (s, 3H, CH_3CO), 1.56–1.51 (m, 2H, $CH_3CH_2CH_2CH_2$), 1.41–1.33 (m, 2H, $CH_3CH_2CH_2CH_2$), 0.93–0.90 (t, 3H, J = 7.3 Hz, CH_3CH_2). MS (ESI) m/z 250 $[M-H]^+$, 252 $[M+H]^+$. Analysis calculated for $C_{14}H_{21}NO_3 \cdot 0.67H_2O$: C, 63.85; H, 8.55; N, 5.32. Found: C, 63.68; H, 8.18; N, 5.11.

5.3.2. 1-(3-((tert-Butylamino)methyl)-4-hydroxy-5-methoxy phenyl)ethanone (***3b***)

Synthesized from **2** (200 mg, 1.03 mmol) and tert-butylamine (150 mg, 2.06 mmol). Brown solid (128 mg, 50% yield), mp: 176–178 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): 7.42 (d, 1H, J = 1.6 Hz, ArH), 7.30 (d, 1H, J = 1.6 Hz, ArH), 4.03 (s, 2H, $ArCH_2N$), 3.92 (s, 3H, OCH_3), 2.53 (s, 3H, CH_3CO), 1.23 (s, 9H, $(CH_3)_3C$). MS (ESI) m/z 250 $[M-H]^+$, 252 $[M+H]^+$. Analysis calculated for $C_{14}H_{21}NO_3$: C, 66.91; H, 8.42; N, 5.57. Found: C, 66.83; H, 8.83; N, 5.67.

5.4. 1-(4-Hydroxy-3-methoxy-5-nitrophenyl)ethanone (***4***)

Apocynin **1** (20 g, 120 mmol), dissolved in glacial acetic acid (300 ml), was cooled to 0 °C in an ice bath with stirring on. Nitric acid (65%, 14 ml, 224 mmol) was then added slowly through a dropping funnel. The reaction mixture was maintained at room temperature for 2 h and was then poured into ice water (250 ml) with stirring on for 10 min. After 30 min of standing, the reaction mixture was filtered through a Buchner funnel and the residue was washed with water, which was recrystallized in 95% ethanol to afford 19.1 g (75% yield) of **4** as a yellow needle crystal, mp: 159–161 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): 11.13 (s, 1H, OH), 8.31 (d, 1H, J = 1.6 Hz, ArH), 7.77–7.76 (d, 1H, J = 1.6 Hz, ArH), 4.01 (s, 3H, OCH_3), 2.63 (s, 3H, CH_3CO). MS (ESI) m/z 212 $[M+H]^+$. Analysis calculated for $C_9H_9NO_5 \cdot 0.2H_2O$: C, 50.33; H, 4.41; N, 6.52. Found: C, 50.63; H, 4.23; N, 6.26.

5.5. 1-(3-Amino-4-hydroxy-5-methoxyphenyl)ethanone (***5***)

To a solution of **4** (19.1 g, 90.0 mmol) in ethanol (300 ml) was added 10% Pd/C (950 mg) and the reaction mixture was hydrogenated by hydrogen over night. The reaction mixture was filtered, and the filtrate was washed with water (50 ml × 5). The organic layer was dried by anhydrous sodium sulfate. Solvent was removed *in vacuo* to afford 13.1 g (85% yield) of **5** as a brown powder, mp: 156–158 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): 7.06 (d, 1H, J = 1.6 Hz, ArH), 7.03 (d, 1H, J = 1.6 Hz, ArH), 5.90 (s, 1H, OH), 3.92 (s, 3H, OCH_3), 2.52 (s, 3H, CH_3CO). MS (ESI) m/z 180 $[M-H]^+$, 182 $[M+H]^+$. Analysis calculated for $C_9H_{11}NO_3 \cdot 0.17H_2O$: C, 58.69; H, 6.20; N, 7.60. Found: C, 58.98; H, 5.64; N, 7.57.

5.6. General procedure for synthesis of compounds ***6a–c***

To a solution of **5** (200 mg, 1.10 mmol), DMAP (134 mg, 1.10 mmol) and triethylamine (500 μl) in THF (20 ml) cooled to 0 °C under nitrogen, was added a solution of excess acid anhydride in THF (5 ml) dropwise. The reaction mixture was allowed to warm to room temperature and stirred for 5 h. THF was removed *in vacuo*, and ethyl acetate (20 ml) was added. The solution was washed with water (5 ml × 3). The organic phase was dried using anhydrous sodium sulfate, and the solvent was removed *in vacuo*. The residue was purified by column chromatography, eluting with ethyl acetate/petroleum ether (1:2, v/v), to afford **6a–c**.

*5.6.1. 4-Acetyl-2-methoxy-6-propionamidophenyl propionate (**6a**)*

Synthesized from **5** (200 mg, 1.10 mmol) and propionic anhydride (715 mg, 5.50 mmol). White powder (274 mg, 85% yield), mp: 151–152 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): 9.42 (s, 1H, NH), 8.19–8.18 (d, 1H, J = 1.6 Hz, ArH), 7.36 (d, 1H, J = 1.6 Hz, ArH), 3.83 (s, 3H, OCH_3), 2.67–2.62 (q, 2H, J = 7.5 Hz, CH_2CH_3), 2.42–2.36 (q, 2H, J = 7.5 Hz, CH_2CH_3), 1.18–1.14 (t, 3H, J = 7.5 Hz, CH_3CH_2), 1.10–1.06 (t, 3H, J = 7.5 Hz, CH_3CH_2). MS (ESI) m/z 292 $[M-H]^+$, 294 $[M+H]^+$. Analysis calculated for $C_{15}H_{19}NO_5$: C, 61.42; H, 6.53; N, 4.78. Found: C, 61.31; H, 6.67; N, 4.55.

*5.6.2. 4-Acetyl-2-butyramido-6-methoxyphenyl butyrate (**6b**)*

Synthesized from **5** (200 mg, 1.10 mmol) and butyric anhydride (870 mg, 5.50 mmol). White powder (307 mg, 87% yield), mp: 138–139 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): 9.43 (s, 1H, NH), 8.14–8.13 (d, 1H, J = 1.6 Hz, ArH), 7.37 (d, 1H, J = 1.6 Hz, ArH), 3.83 (s, 3H, OCH_3), 2.62–2.58 (t, 2H, J = 7.3 Hz, CH_2CO), 2.37–2.33 (t, 2H, J = 7.3 Hz, CH_2CO), 1.73–1.64 (m, 2H, CH_3CH_2), 1.64–1.55 (m, 2H, CH_3CH_2), 1.01–0.97 (t, 3H, J = 7.4 Hz, CH_3CH_2), 0.93–0.89 (t, 3H, J = 7.4 Hz, CH_3CH_2). MS (ESI) m/z 320 $[M-H]^+$, 322 $[M+H]^+$. Analysis calculated for $C_{17}H_{23}NO_5$: C, 63.54; H, 7.21; N, 4.36. Found: C, 63.36; H, 7.47; N, 4.23.

*5.6.3. 4-Acetyl-2-isobutyramido-6-methoxyphenyl isobutyrate (**6c**)*

Synthesized from **5** (200 mg, 1.10 mmol) and isobutyric anhydride (870 mg, 5.50 mmol). White powder (302 mg, 85% yield), mp: 160–162 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): 9.37 (s, 1H, NH), 8.02–8.01 (d, 1H, J = 1.6 Hz, ArH), 7.39–7.38 (d, 1H, J = 1.6 Hz, ArH), 3.83 (s, 3H, OCH_3), 2.93–2.83 (m, 1H, $(CH_3)_2CH$), 2.76–2.66 (m, 1H, $(CH_3)_2CH$), 2.58 (s, 3H, CH_3CO), 1.26–1.24 (d, 6H, J = 7.0 Hz, $(CH_3)_2CH$), 1.10–1.08 (d, 6H, J = 6.8 Hz, $(CH_3)_2CH$). MS (ESI) m/z 320 $[M-H]^+$, 322 $[M+H]^+$. Analysis calculated for $C_{17}H_{23}NO_5$: C, 63.54; H, 7.21; N, 4.36. Found: C, 63.26; H, 7.37; N, 4.21.

*5.7. 1-(3-Amino-4-(tert-butyldimethylsilyloxy)-5-methoxy phenyl) ethanone (**7**)*

To a solution of **5** (500 mg, 2.76 mmol) in dichloromethane (50 ml) cooled to 0–10 °C in an ice bath, was added TBDMS–Cl (830 mg, 5.52 mmol) and imidazole (373 mg, 5.52 mmol). The reaction mixture was stirred at room temperature for 3 h under nitrogen. The reaction mixture was washed with water (10 ml × 3). The organic layer was dried using anhydrous sodium sulfate. Solvent was removed *in vacuo* to afford 900 mg (93% yield) of a while power **7**, mp: 112–114 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): 7.03 (d, 1H, J = 1.6 Hz, ArH), 6.99–6.98 (d, 1H, J = 1.6 Hz, ArH), 3.83 (s, 3H, OCH_3), 2.52 (s, 3H, CH_3CO), 1.01 (s, 9H, $(CH_3)_3C$), 0.21 (s, 6H, $(CH_3)_2C$). MS (ESI) m/z 296 $[M+H]^+$. Analysis calculated for $C_{15}H_{25}NO_3Si$: C, 60.98; H, 8.53; N, 4.74. Found: C, 61.14; H, 8.66; N, 4.77.

*5.8. General procedure for synthesis of compounds **8a–c***

To a solution of **7** (900 mg, 2.56 mmol) in THF was added acid anhydride, DMAP (312 mg, 2.56 mmol) and triethylamine (750 μl). The reaction mixture was stirred at room temperature for 3 h to afford a hydroxyl protected amide intermediate. TBAF (900 mg, 2.56 mmol) was then added and the reaction was maintained at room temperature for 1 h. After solvent was removed *in vacuo*, the residue was purified by column chromatography, eluting with ethyl acetate/petroleum ether (1:1, v/v), to afford **8a–c**.

*5.8.1. N-(5-Acetyl-2-hydroxy-3-methoxyphenyl)propionamide (**8a**)*

Synthesized from **7** (900 mg, 2.56 mmol) and propionic anhydride (665 mg, 5.12 mmol). White powder (275 mg, 42% yield), mp: 150–152 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): 10.03 (s, 1H, NH), 9.26 (s, 1H, OH), 8.10 (s, 1H, ArH), 7.30 (d, 1H, J = 2.0 Hz, ArH), 3.87 (s, 3H, OCH_3), 2.49 (s, 3H, CH_3CO), 2.45–2.40 (m, 2H, CH_2CH_3), 1.11–1.07 (t, 3H, J = 7.5 Hz, CH_2CH_3). MS (ESI) m/z 236 $[M-H]^+$, 238 $[M+H]^+$. Analysis calculated for $C_{12}H_{15}NO_4$: C, 60.75; H, 6.37; N, 5.90. Found: C, 61.15; H, 6.80; N, 5.73.

*5.8.2. N-(5-Acetyl-2-hydroxy-3-methoxyphenyl)butyramide (**8b**)*

Synthesized from **7** (900 mg, 2.56 mmol) and butyric anhydride (808 mg, 5.12 mmol). White powder (289 mg, 45% yield), mp: 200–202 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): 10.07 (s, 1H, NH), 9.31 (s, 1H, OH), 8.09 (s, 1H, ArH), 7.30 (d, 1H, J = 2.0 Hz, ArH), 3.87 (s, 3H, OCH_3), 2.49 (s, 3H, CH_3CO), 2.41–2.37 (t, 2H, J = 7.3 Hz, CH_2CH_2CO), 1.66–1.57 (m, 2H, CH_3CH_2), 0.94–0.90 (t, 3H, J = 7.4 Hz, CH_3CH_2). MS (ESI) m/z 250 $[M-H]^+$, 252 $[M+H]^+$. Analysis calculated for $C_{13}H_{17}NO_4 \cdot 0.25H_2O$: C, 61.04; H, 6.90; N, 5.48. Found: C, 61.07; H, 6.96; N, 5.88.

*5.8.3. N-(5-Acetyl-2-hydroxy-3-methoxyphenyl)isobutyramide (**8c**)*

Synthesized from **7** (900 mg, 2.56 mmol) and isobutyric anhydride (808 mg, 5.12 mmol). White powder (275 mg, 43% yield), mp: 135–136 °C. ^{1}H NMR (DMSO-d_6, 400 MHz): 10.05 (s, 1H, NH), 9.23 (s, 1H, OH), 8.09 (d, 1H, J = 1.6 Hz, ArH), 7.31–7.30 (d, 1H, J = 1.6 Hz, ArH), 3.87 (s, 3H, OCH_3), 2.85–2.74 (m, 1H, $(CH_3)_2CH$), 2.49 (s, 3H, CH_3CO), 1.12–1.10 (d, 6H, J = 6.8 Hz, $(CH_3)_2CH$). MS (ESI) m/z 250 $[M-H]^+$, 252 $[M+H]^+$. Analysis calculated for $C_{13}H_{17}NO_4 \cdot 0.25H_2O$: C, 61.04; H, 6.90; N, 5.48. Found: C, 61.18; H, 6.98; N, 5.76.

*5.9. (Z)-1-(3-(5-Acetyl-2-hydroxy-3-methoxybenzylidene amino)-4-hydroxy-5-methoxyphenyl)ethanone (**9**)*

A solution of **2** (200 mg, 1.03 mmol) and **5** (190 mg, 1.03 mmol) in ethanol was refluxed in an oil bath over night. Solvent was removed *in vacuo* and the product was purified by column chromatography, eluting with ethyl acetate/methanol (10:1, v/v), to afford 210 mg (60% yield) of a red powder **9**, mp: 195–196 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): 8.96 (s, 1H, ArCHN), 7.74 (s, 1H, ArH), 7.62 (s, 1H, ArH), 7.60 (s, 1H, ArH), 7.49 (s, 1H, ArH), 4.03 (s, 3H, OCH_3), 4.01 (s, 3H, OCH_3), 2.63 (s, 3H, CH_3CO), 2.63 (s, 3H, CH_3CO). MS(ESI) m/z 356 $[M-H]^+$. Analysis calculated for $C_{19}H_{19}NO_6 \cdot 1.75H_2O$: C, 58.68; H, 5.83; N, 3.60. Found: C, 58.85; H, 6.06; N, 3.57.

*5.10. 1-(3-(5-Acetyl-2-hydroxy-3-methoxybenzylamino)-4-hydroxy-5-methoxyphenyl)ethanone (**10**)*

To a solution of **9** (200 mg, 0.56 mmol) in methanol (20 ml) cooled to 0 °C, was added sodium borohydride (10.6 mg, 0.28 mmol). The reaction mixture was stirred at room temperature for 2 h. Ice water (10 ml) was added to dissolve the excess sodium borohydride. The reaction mixture was extracted with dichloromethane (5 ml × 3) and the organic layer was dried using sodium sulfate. Solvent was removed *in vacuo* to afford 110 mg (58% yield) of a brown solid **10**, mp: 200–203 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): 7.64–7.63 (d, 1H, J = 1.6 Hz, ArH), 7.50 (d, 1H, J = 1.6 Hz, ArH), 7.13–7.12 (d, 1H, J = 1.6 Hz, ArH), 7.08–7.07 (d, 1H, J = 1.6 Hz, ArH), 4.54 (s, 2H, ArCHN), 3.99 (s, 3H, OCH_3), 3.96 (s, 3H, OCH_3), 2.57 (s, 3H, CH_3CO), 2.53 (s, 3H, CH_3CO). ^{13}C NMR ($CDCl_3$ and CD_3OD, 300 MHz): 199.0 (COMe), 198.5 (COMe), 149.8 (ArC), 147.3 (ArC), 146.4 (ArC), 138.8 (ArC), 136.6 (ArC), 128.4 (ArC), 128.3 (ArC), 125.2 (ArC), 123.6 (ArC), 108.9 (ArC), 106.6 (ArC), 102.2 (ArC), 55.6 (CH_3O), 55.5 (CH_3O), 42.5 (CH_2), 25.5 (CH_3), 25.4 (CH_3). MS (ESI) m/z 360 $[M+H]^+$. Analysis calculated for $C_{19}H_{21}NO_6 \cdot 0.86H_2O$: C, 60.88; H, 6.11; N, 3.74. Found: C, 60.80; H, 6.11; N, 3.87.

5.11. (Z)-N-(5-Acetyl-2-hydroxy-3-methoxybenzylidene)-2-methylpropan-2-amine oxide (**11**)

N-tert-Butylhydroxylamine was first prepared on the day of use by using an activated zinc catalyst. 2-Methyl-2-nitropropane was dissolved in ethanol, and the solution was cooled to 0 °C in an ice bath. Zinc dust (5.89 g, 90 mmol) was added. Glacial acetic acid (10.8 g, 180 mmol) was then added dropwise at such a rate that the temperature maintained below 10 °C with vigorous stirring. Ice bath was removed and the reaction mixture was stirred for 3 h at room temperature. The zinc dust was filtered through a Buchner funnel and the solvent was removed *in vacuo*. The residue was dissolved in methanol for use in condensation with aldehyde **2**. To a solution of **2** (11.6 g, 60 mmol) in methanol (300 ml) was added a solution of N-tert-butylhydroxylamine in methanol (50 ml). The mixture was heated to reflux under nitrogen for 6 h. Solvent was removed and the product was purified by column chromatography, eluting with ethyl acetate/petroleum ether (2:1, v/v), to afford 11.8 g (74% yield) of a yellow powder **11**, mp: 183–184 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): 7.76 (s, 1H, CHNO), 7.53 (d, 1H, $J = 1.6$ Hz, ArH), 7.43–7.42 (d, 1H, $J = 1.6$ Hz, ArH), 3.93 (s, 3H, OCH_3), 2.55 (s, 3H, CH_3CO), 1.65 (s, 9H, $(CH_3)_3C$). ^{13}C NMR (DMSO-d_6, 300 MHz): 196.1 (COMe), 155.2 (ArC), 150.2 (ArC), 138.8 (ArC), 128.0 (CHN), 127.8 (ArC), 117.1 (ArC), 112.7 (ArC), 70.5 (CMe_3), 56.1 (CH_3O), 28.0 ($3 \times CH_3$), 26.5 (CH_3). MS (ESI) m/z 264 $[M-H]^+$, 266 $[M+H]^+$. Analysis calculated for $C_{14}H_{19}NO_4$: C, 63.38; H, 7.22; N, 5.28. Found: C, 63.37; H, 7.37; N, 5.25.

5.12. (R)-N-(5-Acetyl-2-hydroxy-3-methoxyphenyl)-5-(1,2-dithiolan-3-yl)pentanamide (**12**)

To a solution of 5-α-lipoic acid (1.58 g, 7.68 mmol) in DMF (20 ml), were added EDCI (1.46 g, 7.68 mmol) and HOBT (1.03 g, 7.68 mmol). The reaction mixture was stirred at room temperature for 30 min. A solution of **7** (900 mg, 2.56 mmol) in DMF (10 ml) was then added dropwise, and the reaction mixture was maintained 20 h at room temperature. Ethyl acetate (300 ml) was added, and the reaction mixture was washed with ice water (50 ml × 3). The organic layer was dried using anhydrous sodium sulfate and the solvent was removed *in vacuo* to afford a hydroxyl protected amide intermediate. TBAF (900 mg, 2.56 mmol) was then added, and the reaction was maintained at room temperature for 1 h. After removing the solvent, the product was purified by column chromatography, eluting with ethyl acetate/petroleum ether (3:2, v/v), to afford 482 mg (51% yield) of a gray powder **12**, mp: 115–116 °C. ^{1}H NMR ($CDCl_3$, 400 MHz): 8.50 (s, 1H, NH), 7.63 (s, 1H, ArH), 7.37 (d, 1H, $J = 1.6$ Hz, ArH), 3.96 (s, 3H, OCH_3), 3.64–3.58 (m, 1H, SCH), 3.23–3.10 (m, 2H, SCH_2), 2.59 (s, 3H, $COCH_3$), 2.49–2.46 (t, 2H, $J = 6.7$ Hz, CH_2CO), 1.98–1.89 (m, 2H, SCH_2CH_2), 1.86–1.78 (m, 2H, CH_2CH_2O), 1.77–1.72 (m, 2H, CH_2CH_2CH), 1.61–1.53 (m, 2H, $CH_2CH_2CH_2$). ^{13}C NMR ($CDCl_3$, 300 MHz): 197.2 (COMe), 171.7 (CON), 146.8 (ArC), 139.3 (ArC), 129.4 (ArC), 125.2 (ArC), 115.6 (ArC), 105.6 (ArC), 56.3 (2C, CH_3O, CH), 40.2 (CH_2), 38.5 (CH_2), 37.3 (CH_2), 34.6 (CH_2), 28.8 (CH_2), 26.3 (CH_3), 25.1 (CH_2). MS(ESI) m/z 392 $[M+Na]^+$. Analysis calculated for $C_{17}H_{23}NO_4S_2$: C, 55.26; H, 6.27; N, 3.79. Found: C, 55.23; H, 6.45; N, 3.61.

5.13. In vitro macrophage protection assay

RAW 264.7 cells were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (FBS) and 100 units/ml Penicillin–Streptomycin (Gibco). Cell viability was measured by conventional MTT assay. RAW 264.7 cells were seeded (10^4/well, 100 μL) in 96-well plates, and were incubated at 37 °C for 24 h under 5% CO_2. The cells were pre-treated with drugs (0.1, 1, 10, 100 μM) for 1 h. Supernatant was removed, and cells were washed with PBS for three times. Cells were treated with 40 μg/ml of LPS for 24 h. MTT was then added (0.5 mg/ml). After incubation for 4 h, the supernatant was removed and DMSO (150 μl) was added. Absorbance (OD) was recorded at 570 nm using a 96-well plate reader (Bio-RAD, USA).

5.14. Fluorescence-based ROS-scavenging assay

RAW 264.7 cells were seeded in confocal plates. After 24 h, which were treated with various concentrations of apocynin, compounds **10**–**12** (0.1, 1 and 10 μM) in the absence or presence of LPS (1 μg/ml). Then the cells were incubated with 20 μM DCFH-DA for 20 min at 37 °C in CO_2 incubator (5% CO_2). After that, wash with PBS three times, the fluorescence (ex488 nm, em525 nm) was detected by using a laser scanning microscope Carl Zeiss Microscope systems. Fluorescence quantitative determination was assayed by a Berthold TriStar Multimode LB 941 microplate reader.

5.15. Western blot for $P67^{phox}$ expression

RAW 264.7 were pre-treated with various concentrations of apocynin, compounds **10**–**12** (0.1, 1 and 10 μM) for 1 h and stimulated with LPS (1 μg/ml) for 24 h. Cytoplasmic extracts of the cells were resolved on 12% SDS-acrylamide gel by electrophoresis, and transferred to PVDF membranes (PALL). The membranes were blocked in 5% (weight/vol) nonfat milk in Tris-buffered saline–Tbuffer, and then incubated with purified mouse anti-$p67^{phox}$ antibody (BD Transduction, 1:2000), β-actin antibody (Neomakers, 1:2000) at 4 °C over night. After incubation with goat anti-mouse IgG horseradish peroxidase linked antibodies (Santa Cruz, 1:2000) at room temperature for 1 h, the blots were treated with ECL reagents (Pierce) and then exposed to X-ray films. β-Actin was used as an internal loading control.

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