Design, synthesis and evaluation of phenylfuroxan nitric oxide-donor phenols as potential anti-diabetic agents

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ARTICLE INFO
Keywords:
Phenylfuroxan
Phenols
α-glucosidase
Antioxidant
NO releasing

ABSTRACT
Both nitric oxide (NO) dysfunction and oxidative stress have been regarded as the important factors in the development and progression of diabetes and its complications. Multifunctional compounds with hypoglycemic, NO supplementation and anti-oxidation will be the promising agents for treatment of diabetes. In this study, six phenylfuroxan nitric oxide (NO) donor phenols were synthesized, which were designed via a combination approach with phenylfuroxan NO-donor and natural phenols. These novel synthetic compounds were screened in vitro for α-glucosidase inhibition, NO releasing, anti-oxidation, anti-glycation and anti-platelet aggregation activity as well as vasodilatation effects. The results exhibited that compound T5 displayed more excellent activity than other compounds. Moreover, T5 demonstrated significant hypoglycemic activity in diabetic mice and oral glucose tolerance test (OGTT) mice. T5 also showed NO releasing and anti-oxidation in diabetic mice. Based on these results, compound T5 deserves further study as potential new multifunctional anti-diabetic agent with antioxidant, NO releasing, anti-platelet aggregation and vasodilatation properties.

1. Introduction
At present diabetes mellitus (DM) has become a serious clinical problem and public health. DM was associated with vascular complications that resulted in more and more morbidity and mortality, involving a series of risky cardiovascular factors concerned with oxidative stress and the lack of function of NO [1]. This related complications included cardiovascular diseases (CVD), retinopathy, nephropathy, stroke, amputations and renal failure, and so on [2]. It was well-known that long-lasting hyperglycemia was the characteristic of DM, which leads to the overproduction of reactive oxygen species (ROS), such as superoxide anion, hydrogen peroxide, hydroxyl radical, and peroxynitrite [3]. Oxidative stress was the consequence of an increase in the production of ROS and/or a reduction in the anti-oxidant systems. The injury induced by oxidative stress only occurs when the antioxidant defenses are unable to counteract the overproduction of ROS [4]. Moreover, the activation of oxidative stress in turn generated a cascade of deleterious metabolic events such as increased polyol activity, enhanced formation of advanced glycation end-products (AGEs), activation of protein kinase C and nuclear factor κB (NF-κB), and increased hexosamine pathway flux. Additionally, clinical and experimental observations suggest oxidative stress as a leading mechanism in the development of diabetes and its complications [5,6]. It was widely accepted that hyperglycemia and oxidative stress may exaggerate and accelerate protein glycation during postprandial period [7,8]. Free oxygen radicals have been displayed to involve in the formation of advanced glycation end-products (AGEs) [7]. Proteins were modified by glucose variability through the glycation reaction, resulting in the formation of AGEs [8]. As a result, both hyperglycemia and AGEs were regarded as the primary cause of pathologic of diabetic complications.

It was well-known that NO worked as a vascular endothelial protection factor and played an important in the keeping of vascular homeostasis, displaying numerous biological effects [9]. In endothelial dysfunction, the overproduction superoxide anions also can induce changes in the NO signaling system. Related studies reported that superoxide anion could convert NO to peroxynitrite which in reverse can generate two very toxic and reactive radicals: nitrogen dioxide radical and hydroxyl radical [10]. Thus, the short of NO has been considered as a dominant factor in endothelial dysfunction. In addition, studies described that oxidative stress and NO disfunction contribute to the cause of hypertension in humans and animals [11]. Patients with hypertension have impaired antioxidant defense mechanisms both in the
endogenous and exogenous, resulting increased plasma oxidative stress and an exaggerated oxidative stress response to various stimuli.

It is well-known that endothelium in the pathological mechanism of atherosclerotic plaque can produce superoxide anion which is harmful to the body [12]. Under normal physiological conditions, with the help of the superoxide dismutase, superoxide anion can be enzymatically converted to hydrogen peroxide that is very toxic free radical. Low density lipoproteins (LDL) can be modified by oxidative stress with the action of this toxic radical [13]. Research evidence also showed that the production of NO by the endothelium could be reduced in the atherosclerotic vessel [14]. NO plays a role in vascular endothelial protection, indicating a series of biological properties such as mediating vascular relaxation, inhibiting platelet aggregation, eliminating superoxide anions, and exerting anti-inflammation [14–18].

Based on these above, weakening oxidative stress and trying to elevate NO concentrations or to ameliorate responses to NO stimulation have attracted significant interest of therapy of DM and its complications. With regard to these, series of compounds were designed and synthesized that appropriate phenylfuroxan NO-donor was attached to different natural antioxidants such as ferulic acid, caffeic acid, cinnamic acid, 4-hydroxyxynamic acid, 3, 4-dihydroxyphenylacetic acid and 3, 4-dihydroxybenzoic acid (Chart 1). The natural antioxidant molecules possess antioxidant activity and α-glucosidase inhibitory activity [19]. It has been said that radical scavengers and antioxidants prevent the formation of AGEs [20]. Studies have showed that compounds combined antioxidant and anti-glycation activity are more efficient in treating DM [21,22].

These newly synthetic compounds are typical of multi-target agents that a single chemical entity is simultaneously able to regulate multiple targets. At present, it is meaningful to use this kind of agents for the therapy of complicated diseases. Here we cover the conclusive results of the research on the properties of antioxidant, α-glucosidase inhibition, anti-glycation, anti-platelet aggregation, and vasodilatation of six phenylfuroxan NO-donor phenols. Moreover, the hypoglycemic and antioxidant as well as NO releasing were evaluated in vivo. All of the synthetic compounds were obtained through hybridization of natural antioxidant molecules, characterized by widely regulated antioxidant activity, with feasible NO-donor fragment (Fig. 1). The NO-donor fragment employed was phenylfuroxan NO-donor fragment, which reveals broadly modulated anti-platelet aggregation and vasodilator properties in vitro [23].

2. Results and discussion

2.1. Chemistry

The intermediate compound M1 was synthesized reporting in Scheme 1. Cinnamyl alcohol (1) reacted with sodium nitrite in acetic acid to give M1. A generalized synthetic approach to the T1 was also showed in Scheme 1. Intermediate M1 reacted with cinnamic acid (2) in the presence of 4-N,N-dimethylaminopyridine (DMAP) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI) in N,N-dimethylformamide (DMF) solution to give T1. The synthesis of T2 and T3 were completed as depicted in Scheme 2. The hydroxy group of the 4-hydroxycinnamic (3) and ferulic acid (4) were benzyl-protected using benzyl bromide in acetone solution in the presence of potassium carbonate to give the corresponding derivatives 5 and 6. Ester hydrolysis of these products using 6 M sodium hydroxide in THF solution was under 60 °C, then HCl acid gave the related acid 7 and 8. The final protected products 9 and 10 were obtained by reacting with intermediate M1 in the presence of EDCI and DMAP in DMF. Deprotection of these products using titanium tetrachloride (TiCl4) in dichloromethane (CH2Cl2) solution gave the target compounds T2 and T3.

The target compounds T4-T6 were prepared via the synthetic route showed in Scheme 3. The hydroxy groups of the 3, 4-dihydroxybenzoic acid (11), 3, 4-dihydroxyphenylacetic acid (12), and caffeic acid (13) were diphenylmethylene-protected using dichlorodiphenylmethane in methylbenzene under 110 °C to give the corresponding derivatives 14, 15 and 16. The final protected products 17, 18 and 19 were obtained by the action of intermediate M1 on the EDCI and DMAP in DMF. Deprotection of these products using acetic acid-water (4:1) solution under 90 °C afforded target compounds T4, T5 and T6.

2.2. Biological evaluation

2.2.1. Antioxidant activity in vitro

The antioxidant properties of target compounds were measured by scavenging radicals (DPPH and OH) [24,25]. The results are showed in Table 1, which revealed that T4-T6 containing catechol have an excellent scavenging radicals capacities, which were much better than both T2 and T3 containing one phenolic hydroxyl. Comparing T2 and T3, we found that the ortho-methoxy of T3 can enhance the antioxidant
activity of phenolic hydroxyl. T1 with no phenolic hydroxyl did not show antioxidant effect. The results indicated that antioxidant activity is mainly related to the phenolic hydroxyl group and catechol is the important structure for scavenging radicals.

### 2.2.2. α-glucosidase inhibitory activity

The activity of the synthesized compounds was evaluated in vitro against α-glucosidase (maltase and sucrase) from small intestine in rats [26]. Results are expressed as the inhibitor concentration with achieving 50% inhibition of α-glucosidase activity (IC\textsubscript{50}) and reported in Table 2. The IC\textsubscript{50} data demonstrates that the target compounds contained o-catechol group inhibited maltase with IC\textsubscript{50} values ranging from 80.59μM to 136.85μM and sucrase with IC\textsubscript{50} values ranging from 87.18μM to 156.81μM, suggesting that the o-catechol group of target compounds T4, T5 and T6 had significant effects on the inhibitory effects. The results of our present study support the previous researches that polyphenols inhibit α-glucosidase in vitro [19].

![Scheme 1](image1.png)

**Scheme 1.** Synthesis route of intermediate M1 and compound T1. Reagents and conditions: (a) NaNO\textsubscript{2}, AcOH, 0°C; (b) M1, EDCI, DMAP, DMF, r.t.

![Scheme 2](image2.png)

**Scheme 2.** Synthesis route of compounds T2-T3. Reagents and conditions: (a) BnBr, K\textsubscript{2}CO\textsubscript{3}, acetone; (b) 6 M NaOH, THF, 60 °C, HCl; (c) M1, EDCI, DMAP, DMF, r.t.; (d) TiCl\textsubscript{4}, CH\textsubscript{2}Cl\textsubscript{2}.

![Scheme 3](image3.png)

**Scheme 3.** Synthesis route of compounds T4–T6. Reagents and conditions: (a) Ph\textsubscript{2}CCl\textsubscript{2}, PhMe, 110°C; (b) M1, EDCI, DMAP, DMF, r.t.; (c) AcOH: H\textsubscript{2}O = 4:1, 90°C.

### Table 1

<table>
<thead>
<tr>
<th>Compds</th>
<th>DPPH (mM)\textsuperscript{a}</th>
<th>OH (mM)\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>&gt; 10\textsuperscript{b}</td>
<td>&gt; 10\textsuperscript{b}</td>
</tr>
<tr>
<td>T2</td>
<td>0.987</td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>0.177</td>
<td>0.895</td>
</tr>
<tr>
<td>T4</td>
<td>0.0082</td>
<td>0.222</td>
</tr>
<tr>
<td>T5</td>
<td>0.0143</td>
<td>0.231</td>
</tr>
<tr>
<td>T6</td>
<td>0.042</td>
<td>0.223</td>
</tr>
<tr>
<td>T7</td>
<td>0.163</td>
<td>0.895</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The results summarized were the mean values of n = 3 for IC\textsubscript{50} values.

\textsuperscript{b} It indicated that the IC\textsubscript{50} values were not detected even when the maximum concentration was 10 mM.

### 2.2.3. Anti-glycation property

The effects of compounds T1-T6 on AGEs formation are represented in Table 3. T5 and T6 showed higher inhibition of AGEs formation than...
aminoguanidine (AG, IC50 value was 1.510 mM), with IC50 values of 0.386 and 0.175 mM, respectively. T3 and T4 showed the low activity of anti-glycation. T1 and T2 did not show anti-glycation activity. Free radicals have been showed to participate in the formation of AGEs. Antioxidants and radical scavengers inhibit the processes [20]. Our study results support the viewpoint. Moreover, compounds combined antioxidant and anti-glycation properties are more efficient in inhibiting glycation reactions in both early and late stages of AGEs formation [27].

2.2.4. Nitric oxide (NO) releasing activity in vitro

The NO releasing abilities of T1-T6 were determined by Griess assay [28]. As demonstrated in Fig. 2, the active catechol compounds T4-T6 released moderate amount of NO (14.73, 21.30, and 13.54 μM, respectively). And the less active compounds T1-T3 released the similar levels of NO (14.20, 14.80, and 14.35 μM, respectively) under the same conditions. The findings of our study indicate that the phenylfuroxan-phenols hybrids can release moderate amount of NO, which is not significantly affected by the structure of phenolic.

2.2.5. Anti-platelet aggregation activity

The inhibitory activity of the synthetic compounds T1-T6 in vitro on adenosine diphosphate (ADP)-induced platelet aggregation in human platelet rich plasma (PRP) were performed by Born’s turbidimetric method [29]. As showed in Table 4, compound T5 (inhibition rate 20.58% at 0.15 mM) showed slight lower inhibitory effect than aspirin (inhibition rate 28.11% at 0.15 mM) at the same concentration. Compounds T1 (12.66%), T2 (13.78%), T3 (15.46%), T4 (17.66%) and T6 (13.79%) were showed poor inhibitory properties at the same concentration. The anti-platelet aggregation activity of target compounds were associated with their NO releasing abilities. The NO releasing of T5 was the most excellent than other compounds, and its anti-platelet aggregation was also optimal. The results indicated that platelet aggregation inhibitory activity of these compounds may be positively correlated to their NO releasing abilities.

2.2.6. Vasorelaxant property

To study the vasodilative effects of phenylfuroxan NO-donor derivatives, the contraction of the artery ring segments were induced by 60 mM potassium chloride (KCl) Krebs solution. The cumulative concentration of the NO-donor T1-T6 (10−8 to 10−1 M) were added to the arteries when the sustained tension was obtained, respectively [30,31]. Meanwhile, effect of DMSO used as solvent with the same volumes was also evaluated. As demonstrated in Table 5, T1-T6 (10−8 to 10−1 M) induced concentration-dependent relaxations on the mesenteric arteries pre-constricted by KCl 60 mM, respectively. Relaxant effects of T1 (pIC50 = 4.547) and T5 (pIC50 = 4.415) became significant at approximately 10−5 M, which showed to be excellent vasorelaxant effects.

<p>| Table 2 | The α-glucosidase inhibitory properties of the compounds T1-T6 were evaluated against rat intestinal α-glucosidase (maltase and sucrase) in vitro. |</p>
<table>
<thead>
<tr>
<th>Compds</th>
<th>Maltase (μM)a</th>
<th>Sucrase (μM)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>&gt; 2000b</td>
<td>&gt; 2000b</td>
</tr>
<tr>
<td>T2</td>
<td>&gt; 2000b</td>
<td>&gt; 2000b</td>
</tr>
<tr>
<td>T3</td>
<td>301.73</td>
<td>223.36</td>
</tr>
<tr>
<td>T4</td>
<td>123.36</td>
<td>155.03</td>
</tr>
<tr>
<td>T5</td>
<td>80.59</td>
<td>87.18</td>
</tr>
<tr>
<td>T6</td>
<td>136.85</td>
<td>156.81</td>
</tr>
<tr>
<td>Acarbose</td>
<td>5.59</td>
<td>2.31</td>
</tr>
</tbody>
</table>

a The results summarized were the mean values of n = 3 for IC50 values.

b It indicated that the IC50 values were not detected even when the maximum concentration was 2000 μM.

<p>| Table 3 | The effects of target compounds T1-T16 on anti-glycosylation in vitro. |</p>
<table>
<thead>
<tr>
<th>Compds</th>
<th>Anti-glycosylation (mM)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>&gt; 30b</td>
</tr>
<tr>
<td>T2</td>
<td>&gt; 30b</td>
</tr>
<tr>
<td>T3</td>
<td>3.259</td>
</tr>
<tr>
<td>T4</td>
<td>3.037</td>
</tr>
<tr>
<td>T5</td>
<td>0.386</td>
</tr>
<tr>
<td>T6</td>
<td>0.175</td>
</tr>
<tr>
<td>Aminoguanidine</td>
<td>1.510</td>
</tr>
</tbody>
</table>

a The results summarized were the mean values of n = 5 for IC50 values.

b It indicated that the IC50 values were not detected even when the maximum concentration was 30 mM.

<p>| Table 4 | The effects of target compounds T1-T16 on anti-platelet aggregation in vitro. |</p>
<table>
<thead>
<tr>
<th>Compds</th>
<th>Inhibition rate (%)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>12.66</td>
</tr>
<tr>
<td>T2</td>
<td>10.78</td>
</tr>
<tr>
<td>T3</td>
<td>9.46</td>
</tr>
<tr>
<td>T4</td>
<td>17.66</td>
</tr>
<tr>
<td>T5</td>
<td>20.58</td>
</tr>
<tr>
<td>T6</td>
<td>13.79</td>
</tr>
<tr>
<td>Aspirin</td>
<td>28.11</td>
</tr>
</tbody>
</table>

a PRP and tested compounds (0.15 mM) were pre-incubated at 37 °C for 5 min followed by the addition of ADP (5 μL), and the concentrations of target compounds T1-T6 were 0.15 mM. The results were summarized as the mean values of n = 3 for IC50 values.

<p>| Table 5 | Inhibitory effects of compounds T1-T6 on artery contraction induced by KCl 60 mM.a |</p>
<table>
<thead>
<tr>
<th>Compds</th>
<th>Rmax(%)</th>
<th>pIC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>&lt; 10.0</td>
<td>–</td>
</tr>
<tr>
<td>T1</td>
<td>21.4 ± 3.5''</td>
<td>4.547 ± 0.062</td>
</tr>
<tr>
<td>T2</td>
<td>94.3 ± 2.1''</td>
<td>3.538 ± 0.153</td>
</tr>
<tr>
<td>T3</td>
<td>98.2 ± 1.1''</td>
<td>3.778 ± 0.045</td>
</tr>
<tr>
<td>T4</td>
<td>100.8 ± 2.0''</td>
<td>4.286 ± 0.032</td>
</tr>
<tr>
<td>T5</td>
<td>104.8 ± 1.1''</td>
<td>4.415 ± 0.047</td>
</tr>
<tr>
<td>T6</td>
<td>91.0 ± 6.1''</td>
<td>3.986 ± 0.065</td>
</tr>
<tr>
<td>Sodium nitroprusside</td>
<td>89.0 ± 5.1''</td>
<td>6.786 ± 0.119</td>
</tr>
</tbody>
</table>

a Data were expressed as Mean ± SD, n = 8, and analyzed by one-way analysis of variance (ANOVA) test. P < 0.01 vs DMSO.

b Rmax: maximal vasodilative ratio.

Fig. 2. The levels of NO released by T1-T6 were determined in vitro by Griess assay. NO data are expressed as the mean values (n = 3) in μM at each time point.
property. While T2 (pIC₅₀ = 3.538) and T3 (pIC₅₀ = 3.778) were at approximately 10⁻⁴ to 10⁻³ M as well as T4 (pIC₅₀ = 4.286) and T6 (pIC₅₀ = 3.986) were at approximately 10⁻⁴ M. The vasodilative activity of target compounds were similar to their anti-platelet aggregation abilities. The findings indicated that vasodilative activity of these compounds may also be positively correlated to their NO releasing abilities.

2.2.7. Effect of T5 on blood glucose in OGTT mice

As compound T5 showed be strong rat intestinal α-glucosidases (maltase and sucrase) inhibition in vitro. In order to further study the α-glucosidases inhibitory activity of T5, the oral glucose tolerance test (OGTT) was performed in normal mice [32]. Fig. 3(a) showed that the glucose levels of all the groups were significantly increased except blank and acarbose groups between 0 min and 60 min. And then the glucose levels went down rapidly. While the glucose levels of acarbose group slowly reached the top at 60 min, then also slowly went down. During the test, the glucose levels of 50 mg/kg T5 and 100 mg/kg T5 groups still lower than model group. Fig. 3(b) revealed the area under the curve (AUC) of acarbose, T5 (50 mg/kg and 100 mg/kg) showed the significant difference compared to model group, while T5 (50 mg/kg) did not show significant difference, which indicated that T5 had the property of α-glucosidases inhibition. The higher the dosage, the stronger the inhibitory activity. Compound T5 showed dose-dependent effect on the hypoglycemic activity.

2.2.8. Anti-diabetic effects in STZ-induced diabetic mice

2.2.8.1. Hypoglycemic activity. Due to the inhibition of α-glucosidases and anti-glycation in vitro and the good results of OGTT in normal mice, hypoglycemic action was performed in DM mice model [33]. In this section, the STZ-induced ICR diabetic mice with stable hyperglycemic were applied to evaluate the anti-diabetic activity of T5. As showed in Fig. 4, the fasting blood-glucose (FBG) of model group was obviously increased, while the enhanced blood glucose was significantly decreased by 20.1% (P < 0.05) compared with the model group after continuous oral treatment of T5 at doses of 100 mg/kg for 10 days. The positive control acarbose (25 mg/kg) administration apparently reduced the blood glucose levels by 34.6% (P < 0.01) compared with the model group.

2.2.8.2. Plasma NO levels. Effect of T5 on NO levels in STZ-induced diabetic mice after continuous treatment for 10 days was showed in Fig. 5, which exhibited that NO levels in STZ-induced diabetic mice were obviously lower than those in blank group mice, indicating that
intraperitoneal injection with STZ to induce diabetic model led to the abnormality of NO metabolism. However, after continuous administration with T5 for 10 days, T5 was found be able to significantly increase the NO levels in diabetic mice. T5 showed a superior effect on NO supplement than acarbose which displayed no significant difference compared with the model mice.

2.2.8.3. Anti-oxidant activity. As described in Fig. 6, the activity of superoxide dismutase (SOD) was markedly reduced in the plasma of diabetic mice and the production of lipid peroxide product malonaldehyde (MDA) was dramatically increased. Compound T5 showed significantly anti-oxidant activity in vivo, increasing plasma SOD levels by 17.4% ($P < 0.01$) and reducing plasma MDA levels by 35.2% ($P < 0.01$), compared to the model group. The results suggested that catechol structure of T5 was the favorable structure for ameliorating oxidative stress in diabetic mice. Moreover, the potent antioxidant activity of T5 was also confirmed in vivo.

3. Conclusions

In this study, 6 phenyfuroxan-phenols compounds were designed, synthesized and evaluated, which were characterized by a unique multimodal profile. The activity studied in vitro including antioxidant, α-glucosidase inhibition, anti-glycation, NO releasing activity and anti-platelet aggregation as well as vasodilator properties let to identify compound T5 as the potent anti-diabetes and its complications. Phenyfuroxan-phenols compound T5 was found able to reduce the blood glucose in normal and/or diabetic mice. Moreover, has anti-oxidant activity and increases the content of NO in diabetic mice. Compound T5 equipped with good antioxidant activity, which could improve NO deficiency in diabetes. These properties were beneficial for retarding the onset and development of diabetic complications. In brief, the results of the study consumingly indicate that all these properties could be benefit to improve DM and its complications by eliminating ROS, inhibiting AGEs formation, elevating NO level and reducing blood glucose. The strongly effective and reasonably balanced activity of T5 may make it useful in the therapy of DM and its complications.

4. Experimental sections

4.1. Materials

Unless specified otherwise, all starting materials, reagents and solvents were commercially available. The progress of the reactions was monitored by TLC using Qingdao Haiyang Chemical Co. Ltd, HG/T2354-92 Silica Gel GF254. Purifications of compounds were made by flash column chromatography using Qingdao Haiyang Chemical Co. Ltd, silica gel (200 mesh). Melting points were recorded using Tech.

Fig. 6. Plasma levels of SOD and MDA in the diabetes mouse model after intragastrical gavage with T5 (100 mg/kg) and acarbose (25 mg/kg) for 10 days. The results were presented as Mean ± SD (n = 8). Symbols represent significant differences: *$P < 0.05$, **$P < 0.01$ vs blank group, ***$P < 0.01$ vs model group.

4.2. The synthesis of target compounds

4.2.1. 3-(cinnamoyloxy) methyl)-4-phenyl-1, 2, 5-oxadiazole 2-oxide (T1)

Compound intermediate M1 (mp 63–64 °C) was prepared according to the literature procedure [34]. Compound 2 (0.46 g, 3.12 mmol), M1 (0.50 g, 2.60 mmol), EDCI (0.89 g, 4.66 mmol), and DMAP (0.04 g, 0.33 mmol) were dissolved in DMF. The mixture was stirred at r.t. for 12 h. After and that the product was purified by column chromatography (petroleum ether-ethyl acetate, 6:1) to yield T1 as white solid (0.72 g, 64.29%). MP: 86–87 °C, EI-MS (m/z): 322.4 (M+). 1H NMR (400 MHz, DMSO-d6): δ 7.82–7.84 (m, 2H, Ar-H), 7.68–7.71 (m, 2H, Ar-H), 7.63–7.66 (m, 3H, Ar4-H), 7.59–7.63 (d, 1H, J = 15.9 Hz, Ar-CH=CHCOO–), 7.42–7.44 (m, 3H, Ar-H), 6.61–6.65 (d, 1H, J = 15.9 Hz, Ar-CH=CHCOO–), 5.31 (s, 2H, COOH2–) ppm. 13C NMR (101 MHz, DMSO-d6) δ 165.88, 157.50, 146.52, 134.15, 131.87, 131.24, 129.82, 129.36, 128.98, 128.19, 126.23, 116.92, 112.78, 54.73.

4.2.2. (E)-3-(((3-(4-hydroxyphenyl) acryl) oxy) methyl)-4-phenyl-1, 2, 5-oxadiazole 2-oxide (T2)

Compound 7 was prepared according to the literature procedure (Mp: 109–110 °C) [35]. Compound 7 (0.50 g, 1.97 mmol), M1(0.25 g, 1.31 mmol), EDCI (0.45 g, 2.36 mmol), and DMAP (0.03 g, 0.25 mmol) were dissolved in DMF solution. The mixture was stirred at r.t. for 12 h. The product was purified by column chromatography (petroleum ether-ethyl acetate, 8:1) to yield 9 as light green oil (0.525 g). Compound 9 (0.53 g, 1.23 mmol) in CH2Cl2 solution was mixed with TiCl4 (270μL, 2.45 mmol). The mixture was stirred at r.t. for 30 min under the atmosphere of N2. The product was purified by column chromatography (chloroform-methanol, 80:1) to yield T2 as white solid (0.27 g, 65.85%). MP: 152–154 °C, EI-MS (m/z): 338.4
4.3. Biological evaluation

4.3.1. Antioxidant activity

The antioxidant properties of all synthetic compounds were evaluated towards scavenging radicals (DPPH’ and OH’). The assay steps were referred to the literature [24,25].

DPPH’ radical scavenging activity (%) = [(Ao – A1)/Ao] × 100.

where Ao is the absorbance of the solvent control reaction and A1 is the absorbance of varying concentrations of samples. Each absorbance was corrected by using blank solution. All tests were performed as parallel three times. The median efficient concentration (IC50) of each compound was calculated by Excel software. The IC50 value was used to evaluate the DPPH’ radical scavenging activity of compounds. The stronger the antioxidant activity is, the lower the IC50.

4.3.1.1. DPPH radical scavenging activity assay. DPPH’ is based on the nitrogen atom as the centre of the structure and can be existed stably in organic reagents. The absorption of the DPPH’ solution after addition of the antioxidant is reduced at the wavelength of 517 nm. An aliquot (0.5 mL) of 0.08 g/mL DPPH’ solution was combined with 0.5 mL anhydrous alcohol, pH 5.5 and 0.1 M acetate sodium acetate buffer solution and 0.5 mL sample (different concentrations of compounds T1-T6 or anhydrous alcohol reagent). After addition of each component, the solution should be mixed well, then placed at room temperature for 30 minutes in the dark. The absorption is monitored at 517 nm and the DPPH radical scavenging results are expressed as:

4.3.1.2. Hydroxyl radical scavenging activity assay. The hydroxyl radical scavenging activity of target compounds were determined by the Fenton reaction. The assay is based on the reduction of ferrous ions while the colour of solution change. Hydroxyl radicals were generated in the Fe2+–Phenanthroline–H2O2 system (Fenton reaction). The reaction mixture contained 2.1 mM phenanthroline, 50 mM KH2PO4-NaOH buffer (pH 7.4), 2.1 mM freshly prepared FeSO4, 0.03% H2O2(V/V),100 mM ascorbic acid, solvent, and varying concentrations of tested target compounds T1-T6. The 10 mL tubes covering reaction solutions were incubated for 60 min at 37°C. After incubation, the absorbance was measured at 536 nm against an appropriate blank group (without target compounds replaced by an equal amount of distilled water). Hydroxyl radical scavenging percentage was evaluated by comparing the test and blank solutions the results are calculated as follows: Hydroxyl radical scavenging activity (%) = (1 - (A0 – Ab)/A0) × 100.

Thereinto, A0 was the absorbance at 536 nm of the sample reaction which not contains the H2O2 solution and A1 was the absorbance at 536 nm of the control reaction which not contains the H2O2 solution and A0 was the absorbance at 536 nm of the control reaction which contains the H2O2 solution. All absorbances against an appropriate blank mixture (without samples) and all tests were performed as parallel three times. The IC50 value calculated by Excel software was used to evaluate the DPPH’ radical scavenging activity of compounds.

4.3.2. Inhibitory activity towards α-glucosidase

The α-glucosidase inhibitory activity of compounds T1-T6 were assayed towards rat intestinal α-glucosidase included maltase and sucrase. The α-glucosidase from rats small intestinal brush border membranes were prepared according to literatures which were used as the enzyme source of rat intestinal maltase and sucrase [26].

The maltase inhibitory activity: In the measurement of maltase activity, maltose was used as substrate. The α-glucose produced from
hydrolysis of maltose was measured colorimetrically by the instruction of Glucose Test Kit (Nanjing Jiancheng). The enzyme solution 10 μL was respectively preincubated with 50 μL of the target compounds T1-T6 at varying concentrations in phosphate buffer (pH 6.86) at 37 °C for 15 min. Then the substrate (50 mM of maltose, 20 μL) was added, and the reaction was continued to incubate for 1 h, and terminated in a boiling bath. Then 10 μL the above solution was mixed with 500 μL reagent A and 500 μL reagent B, and then the above solution was incubated at 37 °C for 15 min., and then reaction mixture was incubated at 37 °C for 15 min. The amount of o-glucose generated was determined as the absorbance at 505 nm wavelength. The experiment was performed by varying concentrations around the IC50 values calculated by Excel software and the assays were performed three times in parallel. The inhibition ratio was calculated as follows:

\[
\text{Inhibition} \,(\%) = \frac{O - S}{O} \times 100
\]

where \(O\) was the absorbance of negative control, \(S\) was the absorbance of compounds T1-T6 or positive control, \(A\) was the absorbance of blank control. The sucrose inhibitory activity: It was determined by using sucrose as the substrate according to the same procedure of the experiment of maltose.

4.3.3. Anti-glycation activity

The glycation reactions of the body were simulated with bovine serum albumin (BSA)-methylglyoxal (MGO) system and according to the previous literature [27]. The AGEs were determined with the fluorometric spectrophotometry. 2.0 mL BSA (20 mg/mL) was added to 0.1 mol/L MGO (2.0 mL) and shaken well, then added the set different concentrations of target compounds T1-T6 and 2.0 mL PBS (pH 7.4). After that the reaction tubes were incubated at 55 °C for 4 h. Fluorescence intensity was measured by fluorescence spectrophotometer. The emission wavelength was 340 nm and the excitation wavelength was 420 nm. All tests were carried out in five times. The inhibitory rats were calculated as the following formula:

\[
\text{Inhibition} \,(\%) = \frac{F0 - F1}{F0} \times 100
\]

where \(F0\) was the fluorescence intensity of the blank control and \(F1\) was the fluorescence intensity of the sample. The IC50 values were calculated by Excel software.

4.3.4. NO releasing activity

Briefly, 0.1 mM of the target compounds in pH 7.4 phosphate buffer solution (PBS) was incubated under dark conditions at 37 °C for 0–6 h and were sampled every 20 min for the remaining time. The collected samples (1 mL) were mixed with 0.5 mL of sulfanilamide solution and 0.5 mL of N-1-naphthyl-ethylenediamine dihydrochloride and then incubated 10 min at rt., protected from light [36]. The nitrite concentration was determined by ultraviolet spectrophotometry at 540 nm from a standard curve (0–60 μM) derived from NaN3O2.

4.3.5. Anti-platelet aggregation activity

Blood samples were withdrawn from volunteers and mixed with 3.8% sodium citrate solution (9:1, v/v), followed by centrifugation at 810 g for 8 min at rt. After the resulting platelet-rich plasma (PPP) supernatant was collected, the residue was used to obtain platelet-poor plasma (PPP) by centrifuge at 3000g for another 10 min at rt. The PPP was adjusted with PPP to obtain platelet counts of 400–450 × 109 PL/L. Platelet aggregation was measured by Born’s turbidimetric method with a four-channel aggregometer (LBY-NJ Platelet-Aggregometry, Beijing, China) within 3 h after blood collection. For this, PPP (280 μL) was preincubated with solvent, positive control or 0.15 mM compounds (10 μL) for 5 min at 37 °C, followed by the addition of 60 μM of Adenosine 5′-diphosphate sodium salt (USA, Sigma) to induce the platelet aggregation [29]. The maximum aggregation rate (MAR) was recorded within 5 min at 37 °C. The inhibition rates of the tested compounds on platelet aggregation were calculated as the following formula:

\[
\text{Inhibition rate} \,(\%) = \left(100 - \frac{\text{MAR}_{\text{sample}}}{\text{MAR}_{\text{blank}}}\right) 
\]

Wherein MARblank was the maximum aggregation rate of target compounds and MARsample was the maximum aggregation rate of vehicle (distilled water instead of the samples).

4.3.6. Vasorelaxant ability

SD Rats were sacrificed by CO2. The superior mesenteric arteries of rats were gently removed and immersed in cold Krebs solution (mM). The arteries were cut into 2 mm-long ring segments. One wire was connected to a force displacement transducer and attached to an analog-to-digital converter unit (AD Instruments, Hastings, UK). The other was attached to a movable displacement device, allowing fine adjustments of vascular tension by varying the distance between the wires. Then, a sensitive myograph (610 M, Myo Technology A/S, Denmark) was used for recording the isometric tension of the artery ring segments. The mounted artery rings were immersed in temperature-controlled (37 °C) tissue baths filled with 5 mL Krebs solution, which were continuously aerated with 95% O2 + 5% CO2. A normalization procedure was applied to obtain an optimal initial tension for the ring segments. After equilibration for 60 min at the initial tension, the contractile capacity of each artery ring segment was tested by exposure to KCl 60 mM solution. After the sustained tension was obtained, the tested compounds (10−6 to 10−1 M) were added to the baths to induce relaxation, respectively. Krebs solution is composed by NaCl, 119; KCl, 4.6; MgCl2·6H2O, 1.2; CaCl2, 1.5; NaH2PO4·2H2O, 1.2; NaHCO3, 15; glucose 5.6, pH 7.4 [30,31].

4.3.7. Effect of OGTT in normal male

Male ICR mice (20.0 ± 2.0 g) were given adaptive feeding for a week and offered water and normal diet ad libitum. Subsequently, all mice were absolutely dieted for 12 h (allow water) and then the blood glucose was determined by tail cut with hand-held glucometer [32]. All mice were divided into five groups by FBG: blank group (vehicle), model group (vehicle + starch), T5 (50 mg/kg + starch) group, T5 (100 mg/kg + starch) group, acarbose group (acarbose + starch). Compound T5 or vehicle was given half an hour in advance, and then the starch solution (3 g/kg) was given. The blood glucose levels of all the mice were determined respectively with a hand-held glucometer at 30 min, 60 min, 120 min after intragastric gavage with starch. Blood glucose levels were measured with glucose strip by tail cut.

4.3.8. Anti-diabetic effects in STZ-induced diabetic mice

Male ICR mice (20.0 ± 2.0 g) were given adaptive feeding for a week and offered water and normal diet ad libitum. Streptozocin was dissolved in 0.1 M citric acid buffer solution (pH 4.5) and injected by i.p. to all mice after about 16 h overnight fasting. After 72 h, the blood glucose levels were measured after 6 h of fasting. After four days, the blood glucose levels were measured repeatedly. Mice with blood glucose above 16.0 mM were regarded as successful diabetic models and were applied for the experimental test [33]. The STZ-induced diabetic mice were divided into four groups according to their blood glucose levels after 10 days induction of experimental diabetes. Group I received 0.5% CMC-Na as the model group. Diabetic mice in group II were given T5 at the dose of 100 mg/kg. Group II served as the positive control received acarbose (25 mg/kg). Animals in all groups were administrated for 10 days. Within the duration of the test, mice in all groups were allowed food and water ad libitum. The same age normal mice not received STZ were served as the blank group, which received 0.5% CMC-Na. The blood glucose levels of all groups were determined at day 10. Blood samples were collected from the eyeballs of mice at the end of experiment and then were centrifuged at 8000 rpm for 10 min. The levels of plasma NO, SOD and MDA were measured using commercially available kits according to their test instructions.
Acknowledgements

Financial support from the National Natural Science Foundation of China (Grant No. 21172177) is gratefully acknowledged.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2019.103000.

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