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Indazole-Type Alkaloids from *Nigella sativa* Seeds Exhibit Antihyperglycemic Effects via AMPK Activation in Vitro

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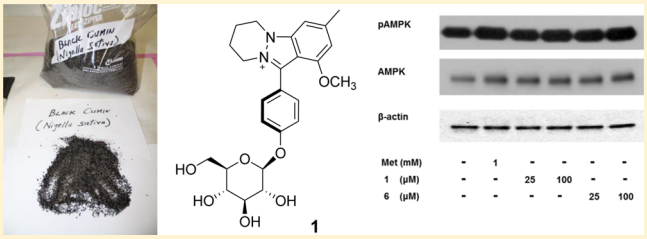
Supporting Information

ABSTRACT: Six rare naturally occurring indazole-type alkaloids including two new compounds, 17-*O*-(β-D-glucopyranosyl)-4-*O*-methylnigellidine (**1**) and nigelanoid (**2**), and four known compounds (**3**–**6**) were isolated from a defatted extract of *Nigella sativa* (black cumin) seeds. 17-*O*-(β-D-Glucopyranosyl)-4-*O*-methylnigellidine (**1**) increased glucose consumption by liver hepatocytes (HepG2 cells) through activation of AMP-activated protein kinase (AMPK). Also, this is the first report of compounds **4** and **6** from a natural source.



AMP-activated protein kinase (AMPK) is an enzyme that plays a key role in cellular energy homeostasis, and the AMPK pathway performs a central function in the regulation of glucose and lipid metabolism. The activation of AMPK can stimulate hepatic fatty acid oxidation and ketogenesis, inhibit cholesterol synthesis, lipogenesis, and triglyceride synthesis, stimulate skeletal muscle fatty acid oxidation and muscle glucose uptake, and modulate insulin secretion by pancreatic beta-cells.[1] Moreover, AMPK has been shown to be a target for antidiabetic drugs, including metformin, and several plant natural products derived from traditional medicines.[1]

Nigella sativa Linn. (Ranunculaceae), commonly known as black cumin, grows in Mediterranean and Middle Eastern countries, South Europe, and Southwest Asia.[2] The seeds of *N. sativa* have been consumed for centuries and are widely used as a spice and traditional medicine for the treatment of various ailments including diabetes.[3] Previous phytochemical investigations of *N. sativa* seeds has led to the identification of oils, saponins, flavonoids, and alkaloids.[4−8] Notably, to date, *N. sativa* is one of only two *Nigella* species reported among all natural sources to contain indazole-type alkaloids.[8]

The seeds of *N. sativa* have been widely studied for their antidiabetic effects,[9] and its most abundant oil constituent, thymoquinone, is implicated as a major bioactive compound responsible for this activity.[10] However, defatted and aqueous extracts of *N. sativa* seeds, from which the aforementioned rare indazole-type alkaloids were isolated,[8] have also been reported to show antidiabetic effects.[11] Furthermore, the in vivo antidiabetic activity of a *N. sativa* seed extract was reported to be mediated through the AMPK pathway,[12] but whether indazole-type alkaloids contribute to these effects is not known. Therefore, a defatted extract of *N. sativa* seeds was investigated to identify bioactive antidiabetic compounds targeting the AMPK pathway. Herein, the isolation and structure elucidation of six rare, naturally occurring indazole-type alkaloids, including two new (**1** and **2**) and four known (**3**–**6**) alkaloids, are reported.

Compound **1**, a yellow, amorphous solid, displayed a molecular formula of $C_{25}H_{31}N_2O_7$, as determined by ^{13}C NMR data and an HRESIMS ion at m/z 471.2129 $[M]^+$ (calcd for $C_{25}H_{31}N_2O_7$, 471.2126) with 12 indices of hydrogen deficiency. In the ^1H NMR data (Table 1), an AA′BB′ spin system with signals at δ_H 7.59 (d, J = 8.3 Hz, H-15, 19) and 7.32 (d, J = 8.3 Hz, H-16, 18), two aromatic protons at δ_H 7.17 (brs, H-7) and 6.75 (brs, H-5), and four methylene signals at δ_H 4.55 (t, J = 6.4 Hz, H-10), 4.43 (t, J = 6.0 Hz, H-13), 2.34 (m, H-11), and 2.21 (m, H-12) were observed, as well as a methyl signal and a methoxy signal at δ_H 2.59 (3H, s) and 3.81 (3H, s), respectively. The ^{13}C NMR (Table 1) and HSQC data revealed the presence of 25 carbon resonances, comprising two methyl, five methylene, 11 methine (six sp^2 and five sp^3), and three quaternary carbons (C-3, C-6, and C-14), an *N*-containing tertiary carbon (C-8), an N,N-disubstituted secondary carbon (C-2), and two oxygenated tertiary carbons (C-4 and C-17).

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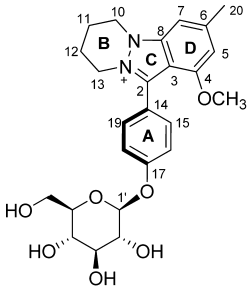

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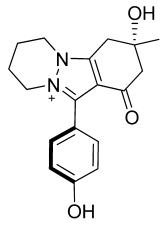

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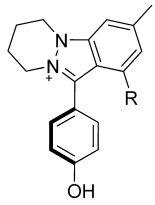

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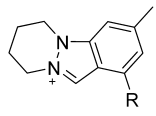
4 R = OCH_3



5 R = OH
6 R = OCH_3

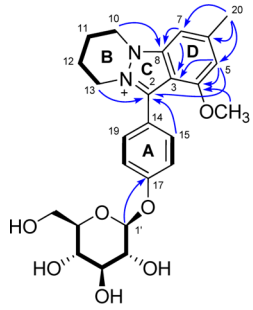
Table 1. ^{1}H NMR and ^{13}C NMR Data of Compounds 1 and 2[a]

	1		2	
no.	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
2	143.2		147.5	
3	109.9		114.7	
4	154.8		189.5	
5	105.6	6.75 brs	51.9	2.86 d (16.4)
				2.68 d (16.4)
6	147.6		71.3	
7	101.0	7.17 brs	34.2	3.19 (2H) s
8	142.6		152.6	
10	46.6	4.55 t (6.4)	47.2	4.51 m
				4.43 m
11	19.3	2.34 (2H) m	18.8	2.27 (2H) m
12	19.8	2.21 (2H) m	19.2	2.17 (2H) m
13	48.4	4.43 t (6.0)	48.3	4.34 t (6.0)
14	118.7		113.8	
15	131.8	7.59 d (8.3)	131.7	7.46 d (8.7)
16	116.2	7.32 d (8.3)	115.3	6.97 d (8.7)
17	159.8		160.7	
18	116.2	7.32 d (8.3)	115.3	6.97 d (8.7)
19	131.8	7.59 d (8.3)	131.7	7.46 d (8.7)
20	21.6	2.59 (3H) s	28.1	1.53 (3H) s
OCH_3	55.0	3.81 (3H) s		
1′	100.4	5.07 d (7.2)		
2′	73.4	3.51 m		
3′	77.0	3.52 m		
4′	70.0	3.42 t (9.1)		
5′	76.6	3.51 m		
6′	61.1	3.94 dd (12.0, 2.0)		
		3.72 dd (12.0, 5.8)		

[a]Data were measured in methanol-d_4 at 500 MHz (^{1}H) and 125 MHz (^{13}C).

The ^{1}H NMR data of **1** also showed the presence of a β-glucopyranose moiety, the anomeric proton of which resonated at δ_H 5.07 (1H, d, J = 7.2 Hz, H-1′). The aforementioned physical data suggested that compound **1** was likely an indazole-type alkaloid, which was supported by the fact that these naturally occurring compounds have been observed only in this genus.[8]

Analysis of the 2D NMR (including ^{1}H−^{1}H COSY, HSQC, and HMBC) data permitted the construction of the structure of compound **1**. After the assignment of all the protons to their bonding carbons by the HSQC data, a hexose moiety (C-1′ to C-6′) and a subunit (C-10 to C-13) (drawn with bold bonds in Figure 1) were established by the ^{1}H−^{1}H COSY data. The



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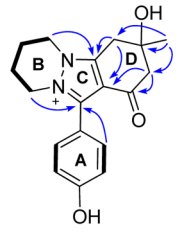

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Figure 1. Key ^{1}H−^{1}H COSY (—) and selected HMBC correlations (H → C) of compounds **1** and **2**.

HMBC correlation (Figure 1) from H-1′ to C-17 (δ_C 159.8) assigned the sugar moiety at C-17 of a *p*-substituted benzene ring (ring A). A tetrasubstituted benzene ring (ring D) was determined by the HMBC correlations from H_3-20 to C-5 (δ_C 105.6), C-6 (δ_C 147.6), and C-7 (δ_C 101.0), from H-5 to C-3 (δ_C 109.9) and C-4 (δ_C 154.8), and from H-7 to C-3 and C-8 (δ_C 142.6), which also allowed for the attachment of Me-20 to C-6. The HMBC correlation from the methoxy protons (δ_H 3.81) to C-4 indicated that a methoxy group was linked to C-4. An sp^2 quaternary carbon, C-2 (δ_C 143.2), was determined to be attached to C-3 and C-14 (δ_C 118.7) based on the HMBC correlation from H-15 to C-2 and the long-range HMBC (J^4) correlation from H-5 to C-2. The chemical shifts of two methylene groups (C-10, δ_C 46.6; C-13, δ_C 48.4) implied that they were linked to nitrogen. The HMBC correlations from H-10 to C-8 and from H-13 to C-2 suggested that CH_2-10 and CH_2-13 were connected to C-8 and C-2 via nitrogen, respectively. The chemical shift of C-2 (δ_C 143.2) required that it was connected to nitrogen to form an azomethine moiety. Finally, it was apparent that the two nitrogens, N-1 and N-9, were connected based on the requirement of the molecular formula and index of hydrogen deficiency. The D-configuration of the glucopyranosyl moiety was determined by acid hydrolysis. The released glucose was identified by co-TLC and comparison of optical rotation with an authentic sample. Thus, the structure of compound **1** was elucidated as 17-*O*-(*β*-D-glucopyranosyl)-4-*O*-methylnigellidine. Compound **1** is the first glucosylated indazole-type alkaloid isolated from a natural source.

Compound **2** (nigelanoid) was obtained as a colorless, amorphous powder with a molecular formula of $C_{18}H_{21}N_2O_3$, as determined by ^{13}C NMR and an HRESIMS ion at m/z 313.1554 $[M]^+$ (calcd for $C_{18}H_{21}N_2O_3$, 313.1547). Analysis of the 1D and 2D NMR data revealed that compound **2** has a similar chemical structure to compound **1**. The striking difference was the absence of the sugar moiety and the two aromatic proton signals in the ^{1}H NMR spectra (Table 1) of compound **2**, compared to **1**. In the ^{1}H NMR spectrum, an AA′BB′ spin system at δ_H 7.46 (2H, d, J = 8.7 Hz, H-15, 19)

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and 6.97 (2H, d, J = 8.7 Hz, H-16, 18) and four methylene signals at δ_H 4.51 (1H, m, H-10), 4.43 (1H, m, H-10), 4.34 (2H, t, J = 6.0 Hz, H-13), 2.27 (2H, m, H-11), and 2.17 (2H, m, H-12) indicated the presence of rings A (*p*-substituted benzene ring) and B. The HMBC correlations (Figure 1) from H_3-20 (δ_H 1.53) to C-5 (δ_C 51.9), C-6 (δ_C 71.3, oxygenated tertiary carbon), and C-7 (δ_C 34.2) indicated a consecutive linkage of C-5, C-6, and C-7 and also allowed for the attachment of Me-20 to C-6. A hydroxy group was assigned to C-6 based on the chemical shift of C-6 (δ_C 71.3). The HMBC correlations from H-5 to C-3 (δ_C 114.7) and C-4 (δ_C 189.5) and from H-7 to C-3 and C-8 (δ_C 152.6) enabled the construction of a six-membered D-ring with an α,β-unsaturated carbonyl moiety. Similarly, the HMBC correlations from H-10 to C-8 and from H-13 to C-2 (δ_C 147.5) were observed. Thus, the planar structure of compound **2** was established. The assignment of the 6*S* absolute configuration was based on the electronic circular dichroism (ECD) data displaying a negative Cotton effect at 281 nm (n → π^* transition) related to the helicity rule of the cyclohexenone ring carbonyl.[13] The structure of compound **2** was thus elucidated as depicted and was assigned the trivial name nigelanoid.

Four known indazole-containing alkaloids were also isolated and identified as nigellidine (**3**),[8b] 4-*O*-methylnigellidine (**4**),[8b] nigeglanine (**5**),[14] and 4-*O*-methylnigeglanine (**6**)[15] on the basis of their NMR and ESIMS data. Notably, compounds **4** and **6** have been previously obtained through synthesis[8b,15] and are being reported here for the first time from a natural source.

Because *N. sativa* seeds have been traditionally used as a treatment for diabetes,[16] the antihyperglycemic abilities of the compounds were evaluated in HepG2 hepatocytes by measuring glucose levels in the media and their effects on AMPK phosphorylation. None of the isolates were cytotoxic to the cells at concentrations reaching 100 μM (see Supporting Information), and therefore they were evaluated at their lowest test concentration (of 25 μM) along with the clinical antidiabetic drug metformin (at 1 mM) to investigate their abilities to regulate glucose consumption in HepG2 cells. All of the isolates significantly decreased glucose levels in the cell supernatants by 8−29%, compared to the control, while metformin decreased glucose levels by 24% (Figure 2A). Notably, 17-*O*-(β-D-glucopyranosyl)-4-*O*-methylnigellidine (**1**) showed the best ability to increase glucose consumption, as evidenced by the fact that it decreased glucose levels by 29% in HepG2 cell culture media. Compound **1** is the only isolate that contains a sugar moiety, and on the basis of this preliminary structure−activity relationship (SAR) observation, it is possible that this structural feature imparts increased glucose consumption ability to this type of compound. However, further SAR studies would be required to confirm this.

To further elucidate whether the isolates increase glucose consumption via AMPK activation, the effects of compounds **1** and **6** on phosphorylation of AMPK level in HepG2 cells were examined. As shown in Figure 2B, Western blot analysis showed that compounds **1** and **6** (at 25 and 100 μM concentrations) increased AMPK phosphorylation (Thr172) in a concentration-dependent manner. Thus, our preliminary in vitro studies support the published animal study wherein the antidiabetic activity of an *N. sativa* seed extract was reported to be mediated through the AMPK pathway.[12]

In summary, previous to this study, only four indazole-type alkaloids had been reported from nature (all obtained from the *Nigella* genus[17]), but none of these compounds were evaluated

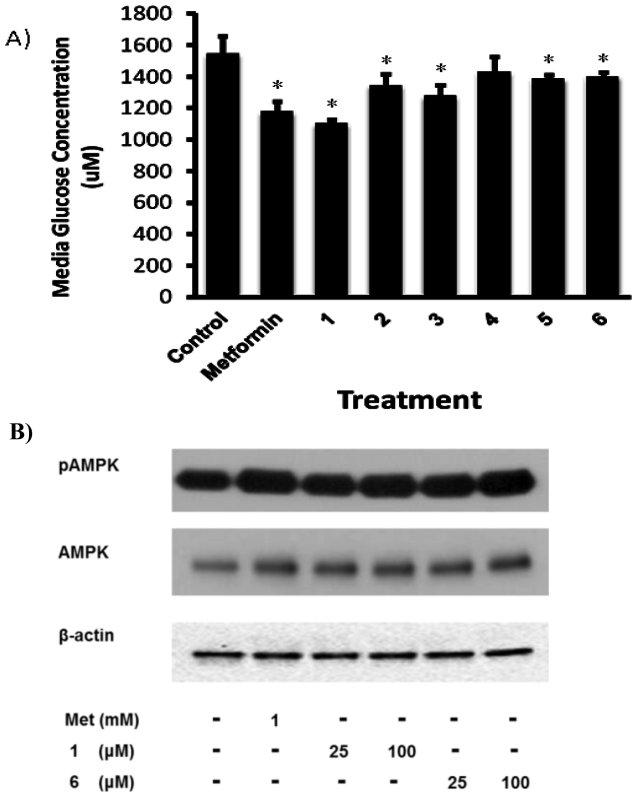

Figure 2. (A) Effect of the isolates (at 25 μM) on glucose consumption in HepG2 cells. HepG2 cells were acclimated to a low-glucose DMEM overnight and then treated with the isolates or metformin in fresh media. Media was collected 7 h later, and glucose concentration was determined. The values are expressed as the means ± SD of three individual samples. *p < 0.05 as compared to the solvent control (0.1% DMSO). (B) AMPK phosphorylation status in HepG2 cells. HepG2 cells were incubated with metformin (Met) and compounds **1** and **6** (each at 25 and 100 μM, respectively) for 24 h, and then cell lysates were collected. Relative p-AMPK and total AMPK were measured by Western blot.

for their biological effects with regard to the antidiabetic properties attributed to *N. sativa* seeds. In the current study, six indazole-type alkaloids (**1**−**6**) were isolated and identified from the defatted extract of *N. sativa* seeds. Among them, 17-*O*-(β-D-glucopyranosyl)-4-*O*-methylnigellidine (**1**) and nigelanoid (**2**) are new compounds, and compounds **4** and **6** are being reported from a natural source for the first time. In addition, 17-*O*-(β-D-glucopyranosyl)-4-*O*-methylnigellidine (**1**) showed more potent ability to regulate glucose consumption than metformin, which was mediated by activation of AMPK. These studies suggest that naturally occurring indazole-type alkaloids could contribute toward the antidiabetic properties reported for *N. sativa* seeds. Also, the compounds identified herein could serve as lead scaffolds for the synthesis of structural analogues with more potent antihyperglycemic activities.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were measured on an Auto Pol III automatic polarimeter (Rudolph Research, Flanders, NJ, USA) at room temperature. The IR spectra were recorded on a Nicolet 380 FT-IR spectrometer. The UV spectra were measured on a Shimadzu UV-2550 UV−visible spectrophotometer. 1D and 2D NMR data were recorded on a Varian 500 MHz instrument with TMS as internal standard. HRESIMS data were acquired using a synapt G2-S QTOF mass spectrometer (Waters,

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Milford, MA, USA). Semipreparative HPLC separations were performed on a Hitachi Elite LaChrom system consisting of an L2130 pump, an L-2200 autosampler, an L-2455 diode array detector, and a Phenomenex Luna C_{18} column (250 × 10 mm, S-5 μm), all operated by EZChrom Elite software. All solvents were of ACS or HPLC grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA) through Wilkem Scientific (Pawcatuck, RI, USA). Silica gel (230−400 mesh, Sorbent Technologies), Sephadex LH-20 gel (Amersham Biosciences), and MCI gel (CHP20P, 63−150 μM, M & M Industries Inc.) were used for column chromatography. Standards of D-glucose and metformin were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Plant Material. *N. sativa* seeds were collected and authenticated by matching the macroscopic and microscopic characteristics to internal monograph and botanical reference standards by Verdure Sciences (Noblesville, IN, USA). A voucher specimen (VS-NSSP-001) has been deposited in the Heber-Youngken Herbarium and Greenhouse, College of Pharmacy, University of Rhode Island.

Extraction and Isolation. The air-dried, ground powder of *N. sativa* seeds (2.0 kg) was extracted with *n*-hexane (4 L × 3) by maceration at room temperature (3 days each time) to afford 298.5 g of crude *n*-hexane extract. The residue was dried and extracted with MeOH (4 L × 3) by maceration at room temperature (3 days each time) to yield 123.1 g of MeOH extract. To further defat this extract, a portion (122.0 g) was reconstituted in MeOH (500 mL) and partitioned with *n*-hexane (500 mL × 3) to yield *n*-hexane (12.3 g) and MeOH (110.0 g) extracts, respectively. The MeOH extract (108.0 g) was chromatographed over a column of MCI gel (MeOH−H_2O, 50:50 to 100:0, v/v) to yield five fractions (A−E). Fraction B (5.2 g) was subjected to separation on a Sephadex LH-20 column (3 × 70 cm), eluted with isocratic MeOH, to obtain three fractions, B1−B3. Fraction B2 (1.3 g) was separated by silica gel chromatography (CC) (4.5 × 40 cm) eluted with a gradient of $CHCl_3$−MeOH (10:1 to 1:1 v/v) to obtain six subfractions, B2a−B2f. Purification of subfraction B2c (194.4 mg) by semipreparative HPLC, eluting with MeOH−H_2O (0−16 min: 10:90 to 41:59; 16−17 min: 41:59 to 100:0; 17−18 min: 100:0; 18−19 min: 100:0 to 10:90; 19−26 min: 10:90; v/v, 3 mL/min), yielded nigeglanine (**5**) (1.8 mg). Fraction D (3.5 g) was purified by semipreparative HPLC by eluting with MeOH−H_2O (0−17 min: 20:80 to 55:45; 17−18 min: 55:45 to 100:0; 18−20 min: 100:0; 20−21 min: 100:0 to 20:80; 21−28 min: 20:80; v/v, 3 mL/min) to yield compounds **1** (9.8 mg), **2** (2.0 mg), nigellidine (**3**) (3.6 mg), 4-*O*-methylnigellidine (**4**) (7.4 mg), and 4-*O*-methylnigeglanine (**6**) (12.6 mg).

*17-O-(β-D-Glucopyranosyl)-4-O-methylnigellidine (**1**):* yellow, amorphous solid; $[\alpha]^{20}_D$ −11 (*c* 0.002, MeOH); UV (MeOH) λ_{max} (log ε) 330 (4.52), 278 (4.39), 228 (3.91) nm; IR ν_{max} 3345, 1618, 1581, 1456, 1382, 860 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 471.2129 $[M]^+$ (calcd for $C_{25}H_{31}N_2O_7$, 471.2126).

*Nigelanoid (**2**):* colorless, amorphous powder; $[\alpha]^{20}_D$ −14 (*c* 0.008, MeOH); UV (MeOH) λ_{max} (log ε) 285 (4.28), 226 (3.95) nm; IR ν_{max} 3415, 1678, 1613, 1598, 1385, 878 cm^{-1}; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 313.1554 $[M]^+$ (calcd for $C_{18}H_{21}N_2O_3$, 313.1547).

Acid Hydrolysis of Compound 1 and Sugar Analysis. Compound **1** (2 mg) was added to a mixture of concentrated HCl (0.5 mL), H_2O (1.5 mL), and dioxane (3 mL) and refluxed for 2 h. After completion of the reaction (monitored by TLC), H_2O was added to the reaction mixture, which was extracted with $CHCl_3$ (3 × 5 mL). The aqueous layer was neutralized with $NaHCO_3$, concentrated to dryness under reduced pressure, and purified by Sephadex LH-20 chromatography to give a sugar fraction. The sugar fraction was determined to be D-glucose by co-TLC and comparison of specific rotation with an authentic sample (R_f = 0.45, $CHCl_3$−MeOH−H_2O, 1:1:0.1, v/v/v, positive value for specific rotation).

Cell Culture. HepG2 cells purchased from American Type Culture Collection (Manassas, VA, USA) were maintained in a high-glucose (4.5 g/L) Dulbecco's modified Eagle's medium culture medium supplemented with 10% FBS, 2 mM glutamine, 1000 U/L penicillin, and 100 mg/L streptomycin at 37 °C, 5% CO_2.

Glucose Consumption Assay. Cells were detached from the culture flask with a solution of 0.25% trypsin and 1 mM EDTA. Trypsin digestion was stopped by the complete culture medium. The cells were seeded into a 96-well plate at a density 4.0 × 10^4 cells/well and cultured for 8 h. The cells were incubated with the low-glucose (1 mg/L) detection medium supplemented with 2 mM glutamine and 1% FBS. After overnight incubation in the detection media, the cells were treated for 7 h with metformin (1 mM) or the isolates (at 25 μM; stock solutions made in DMSO) diluted in the detection medium. The glucose concentration in the medium was determined by a glucose assay kit (Eton Bioscience) as per the manufacturer's instructions. Absorbance was measured at 490 nm, and the assay was performed using 3 replicates per test sample.

Determination of p-AMPK by Western Blot. The cells were seeded into a 6 well plate for 8 h followed by overnight incubation in low glucose media. After 24 h treatment with metformin (1 mM) or the test compounds **1** and **6** (at 25 and 100 μM), total proteins were isolated using RIPA buffer and quantified by the bichonic assay (Pierce, Rockford, IL). Protein homogenates (20 μg/lane) were electrophoretically separated by 8% SDS-PAGE and then transblotted onto Immobilion PVDF membrane (Millipore EMD Corporation, Billerica, MA). Membranes were blocked in 5% non-fat dry milk followed by incubation with primary antibodies p-AMPK, AMPK and β-actin (Cell Signaling Technologies, Danvers, MA) overnight. Membranes were washed 3 times with tris-buffered saline with 0.1% Tween 20 (TBST) followed by incubation with respective secondary horse radish peroxidase-conjugated antibodies (Sigma-Aldrich, St. Louis, MO) for 1 h. After washing the membranes 3 times with TBST, bands were detected on X-ray films using an ECL chemiluminescence detection kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's protocol..

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

The NMR and HRMS spectra of compounds **1** and **2**, as well as the cell viability data are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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