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Isolation, identification, and biological evaluation of HIF-1-modulating compounds from Brazilian green propolis

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ABSTRACT

The tumor microenvironment is characterized by hypoxia, low-nutrient levels, and acidosis. A natural product chemistry-based approach was used to discover small molecules that modulate adaptive responses to a hypoxic microenvironment through the hypoxia-inducible factor (HIF)-1 signaling pathways. Five compounds, such as baccharin (**3**), beturetol (**4**), kaempferide (**5**), isosakuranetin (**6**), and drupanin (**9**), that modulate HIF-1-dependent luciferase activity were identified from Brazilian green propolis using reporter assay. Compounds **3**, **9** and **5** reduced HIF-1-dependent luciferase activity. The cinnamic acid derivatives **3** and **9** significantly inhibited expression of the HIF-1α protein and HIF-1 downstream target genes such as glucose transporter 1, hexokinase 2, and vascular endothelial growth factor A. They also exhibited significant anti-angiogenic effects in the chick chorioallantoic membrane (CAM) assay at doses of 300 ng/CAM. On the other hand, flavonoids **4** and **6** induced HIF-1-dependent luciferase activity and expression of HIF-1 target genes under hypoxia. The contents (g/100 g extract) of the HIF-1-modulating compounds in whole propolis ethanol extracts were also determined based on liquid chromatography–electrospray ionization mass spectrometry as 1.6 (**3**), 14.2 (**4**), 4.0 (**5**), 0.7 (**6**), and 0.7 (**9**), respectively. These small molecules screened from Brazilian green propolis may be useful as lead compounds for the development of novel therapies against ischemic cardiovascular disease and cancer based on their ability to induce or inhibit HIF-1 activity, respectively.

1. Introduction

The tumor microenvironment has attracted much attention as a target for the development of novel cancer treatment strategies because it may be a general target to treat solid tumors regardless of cancer types. Therefore, we have been focusing on the solid tumor microenvironment, characterized by hypoxia, low nutrient availability, and acidosis due to inefficient perfusion for drug discovery of tumor-selective anticancer agents.[1,2] The tumor microenvironment has been recognized as a major factor that not only influences the response to conventional anticancer therapies but also promotes invasion and metastasis.[3,4] In particular, hypoxia is now considered a fundamentally important characteristic of tumor microenvironment. Hypoxia-inducible factor (HIF)-1 has emerged as a key mediator of tumor adaptation and survival in such a deprived microenvironment.[5] HIF-1 modulating compounds should affect adaptive responses in the tumor microenvironment such as angiogenesis, metabolic reprogramming, and metastasis.

Natural products continue to provide promising lead compounds and drug candidates in modern antitumor drug discovery and also serve as useful molecular probes to elucidate signal transduction pathways that control the expression of genes in physiological and pathological states.[6] Most compounds that modulate HIF-1 are natural products or synthetic compounds derived from natural product leads.[7–9] Propolis, produced by honeybees from various plant sources, is a natural resinous hive product and a good source of bioactive polyphenols. It has a broad spectrum of biological activities.[10] Especially, extracts of Brazilian green propolis and Chinese red propolis and their polyphenolic constituents, such as artepillin C and caffeoylquinic acid derivatives, have angiostatic

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effects through suppression of the vascular endothelial growth factor (VEGF) signaling pathway.[11–13] Furthermore, because flavonoids/polyphenols from natural products belong to the largest group of HIF-1 inhibitors,[14–16] we recognized propolis as a useful natural source to be investigated for exploring drug seeds targeting the tumor microenvironment. We therefore evaluated the effects of components of Brazilian green propolis extract, which contains various bioactive polyphenolic compounds, on cellular responses to hypoxia for the development of a novel anticancer drug targeting the tumor microenvironment. In this study, we report the isolation and identification of HIF-1-modulating compounds from Brazilian green propolis extract by using a cell-based luciferase reporter assay[17–19] to assess their effect on HIF-1 transcriptional activity. We also performed chick chorioallantoic membrane (CAM) assay to evaluate anti-angiogenic effects.

2. Results and discussion

2.1. Isolation and identification of HIF-1-modulating compounds

To evaluate the effects of propolis constituents on cellular adaptation to a hypoxic microenvironment, we established human embryonic kidney (HEK) 293 stable reporter cell lines expressing the luciferase coding sequences controlled by a hypoxia response element from the human enolase 1 gene.[20] At first, an ethanol extract was evaluated for a HIF-1-modulating effect in cells exposed to hypoxia (1% O_2). The extract of Brazilian green propolis increased luciferase activity induced by hypoxia by one half compared with that of the control at a concentration of 20 μg/mL (Fig. 1A). Then, the extract was subjected to a silica gel column chromatography to separate 14 fractions (frs.); among them, frs. A3, A8, and A9 exhibited a significantly increased luciferase activity, whereas frs. A6 and A10–A13 resulted in decreased luciferase activity (Fig. 1B). Further the luciferase assay-guided chromatographic separation of these positive fractions (Fig. 2) gave nine known compounds as shown in Figure 3. On the basis of spectral data in comparison with spectra in the literature, the structures of the compounds were identified as lupeol-3-(3′*R*-hydroxy)-hexadecanoate (**1**),[21] artepillin C (**2**),[22] baccharin (**3**),[23] beturetol (**4**),[24] kaempferide (**5**),[25] isosakuranetin (**6**),[26] (*E*)-4-(2,3-dihydrocinnamoyloxy)cinnamic acid (**7**),[27] dihydrokaempferide (**8**),[26] and drupanin (**9**)[28] (Fig. 3; see also the chemical and spectral data in the Supplementary data). All isolated compounds (except for compound **1**, whose solubility in dimethylsulfoxide (DMSO) was extremely poor) were tested for their effects on HIF-1 transcriptional activity under hypoxic conditions at a concentration of 50 μM (Fig. 4). Treatment of cells with compound **3**, **5**, or **9**, which were

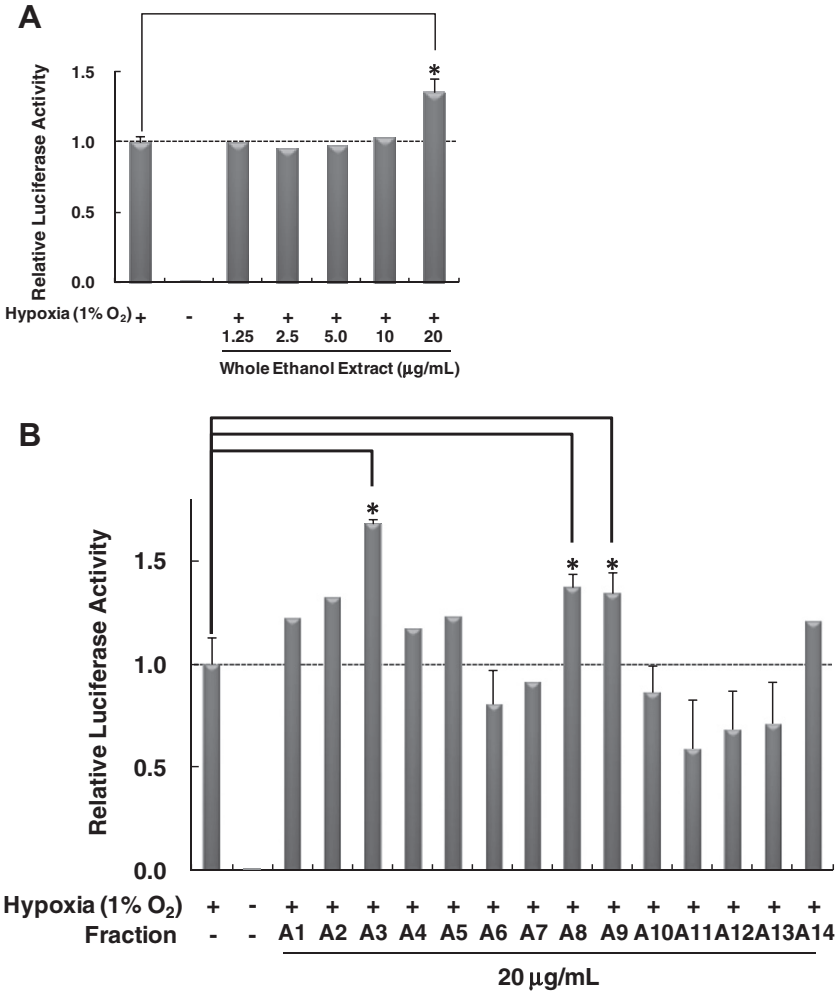


Figure 1. Effect of the extract and the separated fractions of Brazilian green propolis on HIF-1 transactivation. (A) The whole ethanol extract of Brazilian green propolis was evaluated by luciferase assay. (B) Fourteen fractions separated by silica gel column chromatography described in Figure 2 were each evaluated at 20 μg/mL. Averages from one representative experiment performed in triplicate are shown, and the error bars indicate standard deviations. An asterisk indicates the statistical significance compared with the hypoxic control; $^*p$ <0.05.

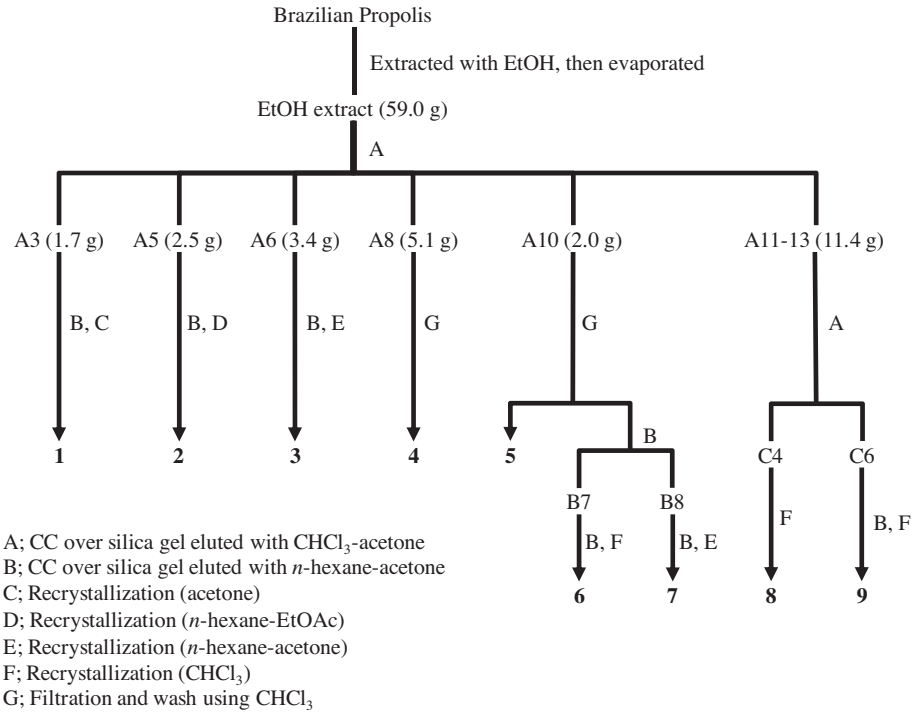


Figure 2. Flowchart for the isolation of chemical constituents from Brazilian green propolis.

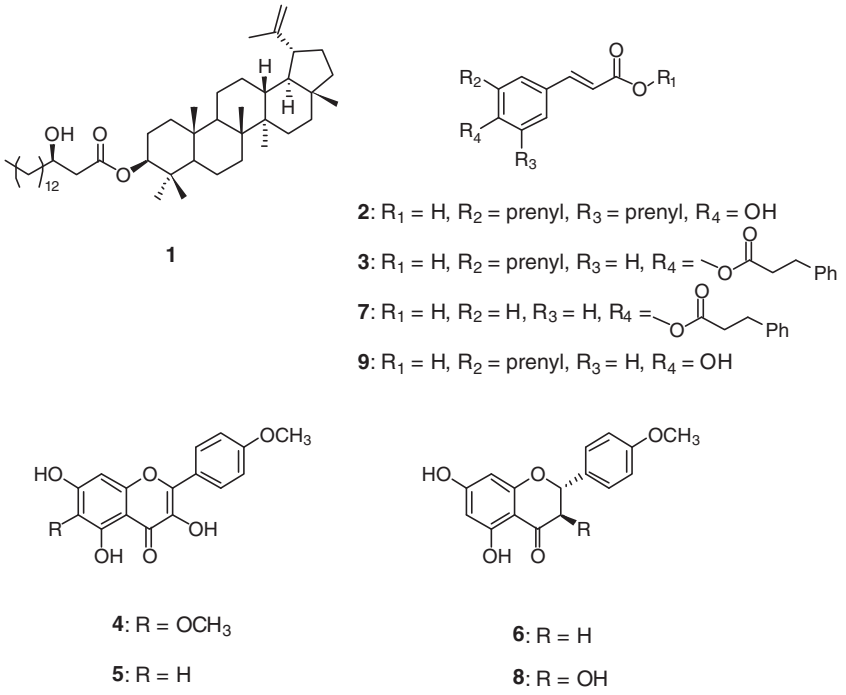


Figure 3. Structures of the compounds isolated from Brazilian green propolis.

isolated from fractions with inhibitory effects, resulted in significantly reduced luciferase activity. On the other hand, treatment of cells with flavonoids **4** and **6** resulted in a significant increase in luciferase activity under hypoxic condition. Furthermore, these HIF-1-modulating effects occurred in a dose-dependent manner (Fig. 5). The cytotoxicity of the active compounds was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and clonogenic assays. At the maximum concentration of the compounds that dissolved in the medium, all active compounds had weak or no cytotoxic effects (Table 1). Although the MTT and clonogenics assays were also performed under hypoxic conditions, there were no differences in the results compared with those observed under normoxic conditions (Table 1).

Among the 4-hydroxycinnamic acid derivatives **2**, **3**, **7**, and **9**, compounds **3** and **9** showed HIF-1 inhibitory effects. To evaluate the pharmacophore of the 4-hydroxycinnamate derivatives, we

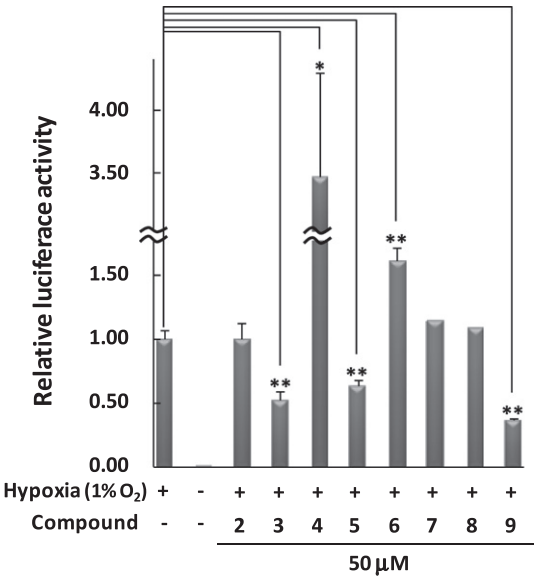


Figure 4. Effects of isolated compounds from Brazilian green propolis on hypoxia-induced HIF-1-dependent luciferase reporter gene activity. Compounds **2**–**9** were examined at a concentration of 50 μM. Mean data from one representative experiment performed in triplicate are shown, and the error bars indicate standard deviations. The asterisks indicate the statistical significance compared with the hypoxic control; $^{*}p$ <0.05, $^{**}p$ <0.01.

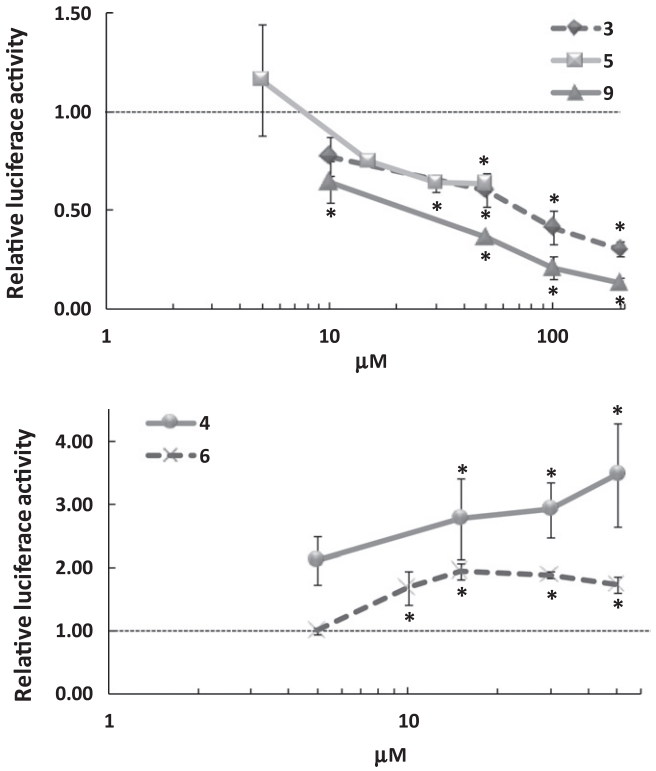


Figure 5. Dose–response relationship of **3**–**6** and **9** in the HIF-1-dependent reporter assay. Data shown are means from one experiment performed in triplicate, and the bars represent standard deviations. An asterisk indicates statistical significance compared with the hypoxic control; $^{*}p$ <0.05.

modified **9** to obtain the 4-methoxy-3-prenyl cinnamic acid methyl ester (**10**), 4-hydroxy-3-prenyl cinnamic acid methyl ester (**11**), and 4-methoxy-3-prenyl cinnamic acid (**12**), and synthesized cinnamic acid derivatives **17** and **18** from commercially available reagents, respectively (Table 2; see also Scheme S1 in Supplementary data). The methyl ester derivatives **10** and **11** bearing conjugated ester as a Michael acceptor were highly cytotoxic, also as expected from their higher hydrophobicity as evidenced by the *c* log *D* values (4.31 and 4.03, respectively) (Table 2). Methyl ether **12** was more hydrophilic and non-cytotoxic at a maximum concentration of 100 μM. But, it did not show a significant inhibiting effect at a concentration of 50 μM in the HIF-1-dependent reporter assay, suggesting the hydrogen donor ability of 4-hydroxy group was necessary for their activity. Compound **3** may become active after metabolic hydrolysis of dihydrocinnamate ester to reveal 4-hydroxyl group. 3-Prenyl group was also important because neither corresponding analogs with 3-allyl (**17**), *t*-butyl (**18**) group nor 3-desprenyl analog (**13**) showed any HIF-1 inhibition. Compound **9** showed the most potent HIF-1 inhibitory effect among all polyphenols tested in this study (Table 2). These data suggested that hydrogen bond donor abilities of phenol, conjugated carboxylic acid moiety of 4-hydroxycinnamic acids, and monoprenyl group were important for HIF-1 inhibitory effect.

Next, the essential moiety for modulating effects of flavonoids (**4**–**6** and **8**) on HIF-1 activity are quite interesting. The common feature among them is the chelating moiety at 5-hydroxyl 4-oxo group.[29] It is known that flavonoids with such a coordination site induce accumulation of HIF-1α under normoxic condition due to their iron-chelating properties. They would inhibit HIF-1α prolyl hydroxylases (PHDs), which play a key role for HIF-1α degradation and contain Fe(II) in their catalytic center, resulting in stabilization of HIF-1α in normoxia.[30,31] On the other hand, Hasebe et al. showed that some flavonoids including quercetin and homoisoflavonoids which possessed 5-hydroxyl 4-oxo group inhibited HIF-1-dependent reporter activity under hypoxic condition.[15] These apparently discrepant reports indicate that the HIF-1 modulating effects of the flavonoids depend on oxygen concentration. Interestingly, we found that flavonoids **4** and **6** enhanced HIF-1 transcriptional activity even under hypoxic condition. Because PHDs were inactivated under hypoxic condition, their inducing effects on HIF-1 transcriptional activities in hypoxia may not be caused by inhibition of PHDs but by some direct affects on the structure or functions of HIF-1α protein. While, compound **5** reduced HIF-1-dependent luciferase activities moderately as in the case of quercetine.[15] Common structure of HIF-1 inhibiting flavonoids shown in the previous report[15] and **5** is 3,5,7-trihydroxy-1,4-benzopyrone moiety. In contrast, with the presence of only 6-methoxy group, flavonoid **4** showed a significant inducing effect of HIF-1 transcriptional activities.

Consequently, hydroxycinnamic acid and flavonoid seem to be interesting and unique scaffolds of HIF-1 modulator, while it is necessary to further investigate the structure-activity relationship.

2.2. Inhibition of expression of HIF-1α protein and HIF-1 target genes

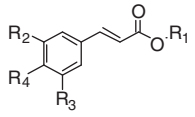
Immunoblot analyses of the HIF-1α protein expression in human colorectal cancer cells treated with compounds **3** and **9** are shown in Figure 6A and B. HCT116 cells were exposed to the compounds under normoxic (20% O_2) or hypoxic conditions for 4 h. Compounds **3** and **9** reduced the expression of the HIF-1α protein induced by hypoxia in a dose-dependent manner (Fig. 6A and B) at concentrations that are comparable with those that inhibited HIF-1-dependent luciferase activity as already shown in Figure 5. Their effects on HIF-1-dependent induction of the glucose transporter 1 (*GLUT1*), hexokinase 2 (*HK2*) and *VEGFA* gene expression in hypoxic HCT116 cells were also investigated by real-time reverse transcription-polymerase chain reaction (RT-PCR). Treatment of cells with compounds **3** and **9** for 24 h equally significantly reduced the

Table 1
Cytotoxicity and hydrophobicity of isolated compounds under hypoxic (1% O_2) or normoxic (20% O_2) conditions

Compounds	Concn (μM)	MTT assay[a]		Clonogenic assay[a]		*c* log *D* (pH 7.0)[c]
		Hypoxia[b]	Normoxia	Hypoxia[b]	Normoxia	
3	200	69.4 ± 2.0	58.2 ± 4.7	74.0 ± 6.2	74.8 ± 2.3	3.75
4	50	86.8 ± 3.3	69.0 ± 2.4	90.3 ± 6.6	96.8 ± 2.6	3.21
5	50	173.3 ± 25.6	102.5 ± 16.0	87.6 ± 6.8	79.9 ± 5.2	2.96
6	50	ND[d]	103.4 ± 9.7	104.7 ± 7.3	99.0 ± 7.1	2.57
9	200	146.0 ± 4.6	127.1 ± 9.4	101.2 ± 2.0	105.8 ± 2.2	1.17

[a] Viability (%) at indicated concentrations.
[b] 1% O_2.
[c] Caluculated by Accord for Excel ver. 7.1.5 (Accelrys, Inc.).
[d] Not determined.

Table 2
Biological activity and hydrophobicity of cinnamic acid derivatives



Compounds	R_1	R_2	R_3	R_4	HIF-1-dependent[a] Luciferase activity	MTT assay[b] Viability (μM)	c log *D* (pH 7.0)[c]
2	H	Prenyl	Prenyl	OH	1.09 ± 0.03	53.3[d] (200)	2.53
3	H	Prenyl	H		0.52 ± 0.06 77.4 ± 25.1[e]	58.2 (200)	3.19
7	H	H	H		1.08	86.0[d] (100)	1.27
9	H	Prenyl	H	OH	0.36 ± 0.03 25.6 ± 12.4[e]	127.1 (200)	1.12
10	CH_3	Prenyl	H	OCH_3	1.17 ± 0.23[f]	37.6 ± 3.7[e]	4.31
11	CH_3	Prenyl	H	OH	ND[g]	10.6 ± 1.2[e]	4.03
12	H	Prenyl	H	OCH_3	0.82 ± 0.08	95.7(100)	1.63
13	H	H	H	OH	0.93 ± 0.07	99.3 (100)	−0.59
17	H	Allyl	H	OH	0.90 ± 0.10	98.4 (100)	0.73
18	H	*t*Bu	*t*Bu	OH	0.77 ± 0.04	108.4 (100)	2.84

[a] Show the ratio of hypoxic control using HEK293 p2.1 #3 cells at concentration of 50 μM.
[b] Using HCT116 cells.
[c] Caluculated by Accord for Excel ver. 7.1.5.
[d] Using HEK293 p2.1 #3 cells.
[e] IC_{50} valus (μM).
[f] Activity at 30 μM.
[g] Not determined.

expression of *GLUT1*, *HK2*, and *VEGFA* mRNA induced by hypoxia at concentrations of 50 μM (Fig. 6C–E). On the other hand, the HIF-1-inducing flavonoids **4** and **6** showed a tendency to induce all HIF-1 target genes both under hypoxic and normoxic conditions. Compound **5** did not show significant suppression of the HIF-1α protein (data not shown), but *GLUT1* induction by hypoxia was reduced as much as those by compounds **3** and **9**. However, expression of the *VEGFA* gene was most potently induced by compound **5** among them by twice as much as that of hypoxic control. As mentioned above, the effect of flavonoid **5** on the expression of HIF-1 related genes were not consistent with those of cinnamate analog HIF-1 inhibitors (**3** and **9**), suggesting that flavonoid and hydroxycinnamic acid might modulate HIF-1 via different pathway based on their unique scaffold.

2.3. Angiogenesis inhibition by CAM assay

The anti-angiogenic effects of the compounds were evaluated in vivo by a CAM assay. Because artepillin C (**2**) was previously identified to be an angiogenesis inhibitor,[11] it was also examined. As shown in Figure 7, the hydroxycinnamic acid derivatives **3** and **9** suppressed angiogenesis similarly to that of artepillin C (**2**) at concentrations of 300 ng/CAM. However, compound **2** did not inhibit HIF-1-dependent luciferase activity (Fig. 4). On the other hand, flavonoids **4**, **5**, and **6** did not show significant effects (Table 3).

2.4. Quantitative analysis of Brazilian green propolis by liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS)

To estimate the contents of the HIF-1-modulation constituents from Brazilian green propolis, quantitative analysis by LC–ESI-MS was performed. Reversed-phase high performance LC of the Brazilian green propolis ethanol extract gave good separation of **3**–**6** and **9** using the modified conditions in de Sousa's method,[32] and the compounds were identified by ESI-MS (Fig. 8; see also ESI-MS data in the Supplementary data). Their contents (g/100 g extract), estimated using an internal standard calibration method, were 1.6 (**3**), 14.2 (**4**), 4.0 (**5**), 0.7 (**6**), and 0.7 (**9**), respectively. Veratraldehyde was used as the internal standard. Compound **4**, which significantly enhanced HIF-1 transcriptional activity (Fig. 4), had the

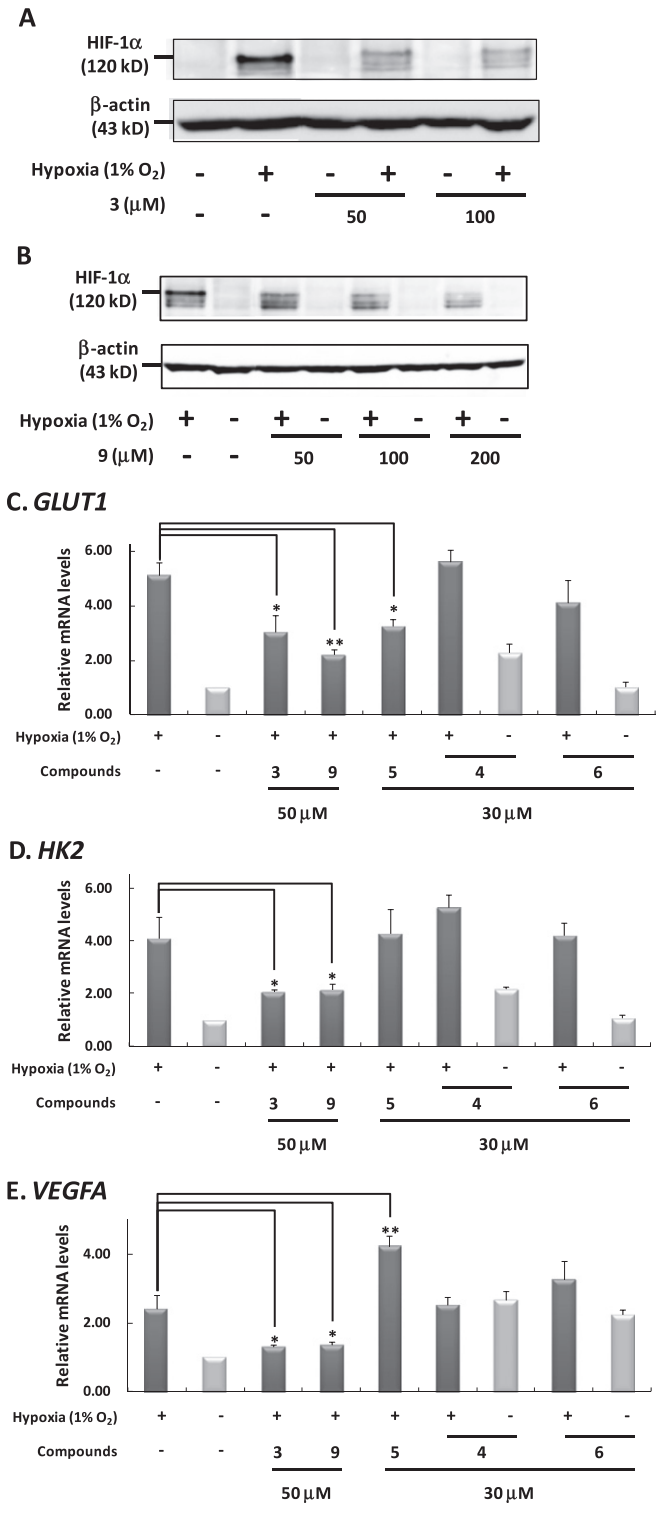


Figure 6. Effects of the test compounds on HIF-1 pathway in HCT116 human colorectal cancer cells. HIF-1α protein expression availability in HCT116 cells. Immunoblot assays of HIF-1α and β-actin proteins following 4 h treatment with the test compounds, **3** (A), **9** (B), and under hypoxic (1% O_2) or normoxic (20% O_2) conditions. Compounds **3**–**6** and **9** regulated the induction of the HIF-1 target gene expression in HCT116 cells. Quantitative real-time RT-PCR analysis of *GLUT1* (C), *HK2* (D), and *VEGFA* (E) mRNA levels upon treatment with each compound for 24 h under hypoxic or normoxic conditions. The data (mean ± SD) were normalized to an internal control (*18S rRNA*), and the relative expression levels were determined by the $\Delta\Delta C_T$ method (see Section 4.3.8). The *p* values are shown where there is a statistically significant difference ($^{*}p$ <0.05, $^{**}p$ <0.01) between the hypoxic control and the compound-treated samples.

highest proportion in the extract. Thus, compound **4** may have significantly contributed to the net increased HIF-1-dependent luciferase activity of the whole extract (Fig. 1A).

3. Conclusion

We have identified five compounds from Brazilian green propolis that modulate HIF-1 activity. The results of biological evaluations of the HIF-1-modulating compounds are summarized in Table 3. None of the compounds showed significant cytotoxicity. We also found that the hydroxycinnamic acid derivatives **3** and **9** from Brazilian green propolis inhibited not only HIF-1 transcriptional activity but also the hypoxia-induced expression of the HIF-1α protein and downstream target genes, such as *GLUT1*, *HK2*, and *VEGFA*. Furthermore, the HIF-1 inhibitory compounds showed angiogenesis inhibition. Additional studies are warranted to determine whether they have antitumor effects in xenograft models as demonstrated for other HIF-1 inhibitors.[17–19] On the other hand, the flavonoid **4** induced HIF-1 transcriptional activity under normoxia as well as hypoxia. This compound may be useful in the treatment of ischemic cardiovascular disease.[33]

4. Materials and methods

4.1. General experimental procedures

^{1}H and ^{13}C NMR spectra were recorded using a JEOL JNM-AL400 or JNM-ECA500 spectrometer at 400 or 500 MHz (^{1}H NMR) and 100 or 125 MHz (^{13}C NMR) in deuterated chloroform ($CDCl_3$), DMSO-d_6 or acetone-d_6. Chemical shifts for ^{1}H NMR were referenced to tetramethylsilane (0.00 ppm). Chemical shifts for ^{13}C NMR were calibrated to the solvent signals ($CDCl_3$: 77.0 ppm, DMSO-d_6: 39.5 ppm, and acetone-d_6: 29.8 ppm). The electron impact (EI)-MS, high resolution (HR)EI-MS, direct analysis in real time (DART)-MS and HRDART-MS measurements were performed on a JEOL JMS-SX102A and JMS-T100TD. Column chromatography was performed with normal-phase silica gel (AP-300; Daico Trading Co., Ltd.). LC-MS spectra were measured using a HP 1100 Series and a HP LC/MSD (Agilent Technologies). Unless otherwise noted, all chemicals were obtained from commercial sources and used without further purification.

4.2. Chemicals

4.2.1. Materials

Brazilian green propolis ethanol extract (#Y080527, collected in Minas Gerais, Brazil) was obtained from Yamada Bee Farm (Okayama, Japan). 4-Hydroxycinnamic acid (**13**) was purchased from Sigma-Aldrich Japan and used for biological studies as well as starting material of **17** without further purification.

4.2.2. Isolation and identification

The residue from the propolis ethanol extract (59.0 g) was first applied to silica gel column eluted with chloroform ($CHCl_3$)–acetone ($CHCl_3$→20:1→5:1→1:1) and acetone (each volume; 2 L, Column A, Fig. 2) to separate 14 fractions. Fraction A3 (1.7 g) was purified by repeated silica gel chromatography using *n*-hexane–acetone (40:1→10:1→ 5:1→1:1) and recrystallized (acetone) to give **1** (153.6 mg). Fraction A5 (2.5 g) was purified by silica gel column chromatography using *n*-hexane–acetone (20:1→10:1→ 5:1→1:1) and recrystallized (*n*-hexane–ethyl acetate) to give **2** (207.4 mg). Fraction A6 (3.4 g) was separated by silica gel column chromatography using *n*-hexane–acetone (20:1→10:1→5:1→1:1) and recrystallized (*n*-hexane–acetone) to give **3** (102.4 mg).

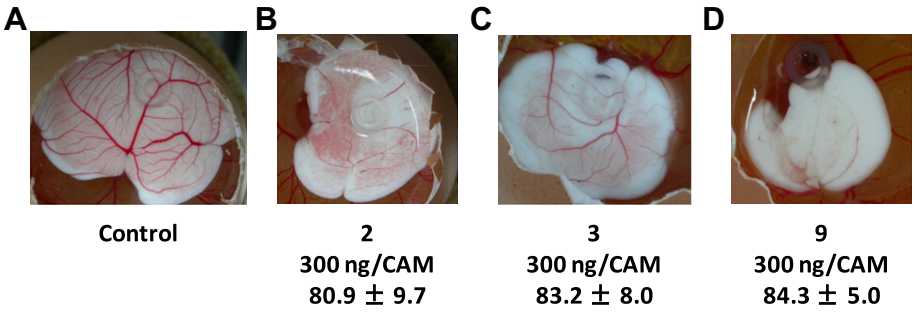


Figure 7. Anti-angiogenic effects of compounds **2**, **3**, and **9**. Each compound was loaded on CAMs of chick embryos at 5 days of age. After 48-h incubation, fat emulsion was injected under the CAMs to make the vascular network clear for photography. (A) control (2% DMSO); (B) **2** (300 ng/CAM); (C) **3** (300 ng/CAM); and (D) **9** (300 ng/CAM). Each angiogenesis inhibition ratio (%; mean ± SD) is also shown under photography.

Table 3
Summary of biological evaluations of HIF-1-modulating compounds from Brazilian green propolis

Compounds	Reporter assay[a]	Western blot	Real-time RT-PCR			CAM assay[b]
	HIF-1	HIF-1α	*GLUT1*	*HK2*	*VEGFA*	
3	↓[c] 77.4 ± 25.1	↓ 50 μM	↓ 50 μM	↓	↓	83.2 ± 8.0
4	↑[d]	±[e] 30 μM	↑[f] 30 μM	↑[f]	↑[g]	46.7 ± 8.1
5	↓	± 30 μM	↓ 30 μM	±	↑	39.5 ± 9.6
6	↑	ND[h]	± 30 μM	±	↑[f]	38.1 ± 5.7
9	↓ 25.6 ± 12.4	↓ 50 μM	↓ 50 μM	↓	↓	84.3 ± 5.0

[a] IC_{50} value (μM).
[b] Inhibition % of angiogenesis at 300 ng/CAM.
[c] ↓, Suppression under hypoxia.
[d] ↑, Induction under hypoxia.
[e] ±, Difference is not significant.
[f] Under hypoxia and normoxia.
[g] Under normoxia.
[h] Not determined.

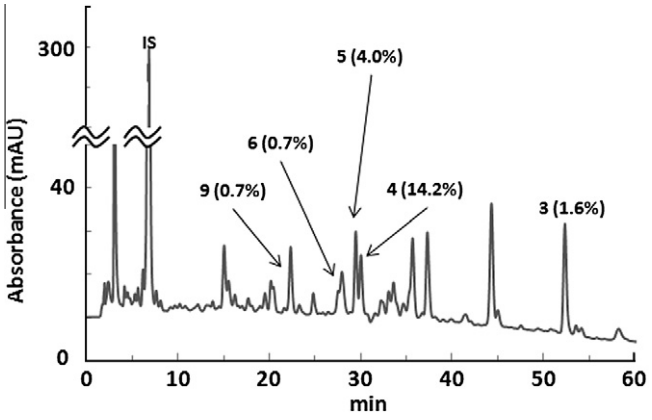


Figure 8. Chromatographic profile of the Brazilian green propolis at 280 nm absorption revealed the following compounds (g/100 g) in extract of Brazilian green propolis: **3**, 1.6; **4**, 14.2; **5**, 4.0; **6**, 0.7; and **9**, 0.7. Internal standard (IS): veratraldehyde.

Fraction A8 (5.1 g) was suspended in $CHCl_3$, and the residue was filtered and washed to give **4** (30.7 mg). Fraction A10 (2.0 g) was suspended in $CHCl_3$, the residue filtered and washed to give **5** (350.3 mg), and then subjected to silica gel column chromatography using *n*-hexane–acetone (10:1→5:1→1:1) (each volume; 200 mL, Column B) to give 10 fractions. Fraction B7 (145.4 mg) was purified by silica gel column chromatography using *n*-hexane–acetone (5:1→1:1) and recrystallized ($CHCl_3$) to give **6** (20.0 mg). Fraction B8 (132.5 mg) was recrystallized (*n*-hexane–acetone) to give **7** (10.4 mg). Fractions A11–13 (11.4 g) were separated by silica gel chromatography using $CHCl_3$–acetone (40:1→5:1→1:1) (each volume; 1 L, Column C) to give 7 fractions. Fraction C4 (712.0 mg) was recrystallized ($CHCl_3$) to give **8** (180.0 mg). Fraction C6 (4.6 g) was purified by silica gel column chromatography using *n*-hexane–acetone (5:1→1:1) and recrystallized ($CHCl_3$) to give **9** (188.0 mg). The structures of the isolated compounds were identified on the basis of physicochemical properties and spectral data comparison with spectra published in the literatures (see the chemical and spectral data in the Supplementary data).

4.2.3. Syntheses of cinnamic acid derivatives

4.2.3.1. 3-(4-Methoxy-3-prenylphenyl)acrylic acid methyl ester (10)[34]. Compound **9** (26.2 mg, 0.11 mmol) was dissolved in 0.5 mL of methanol, then $TMSCH_2N_2$ (0.5 mL) was added and stirred at room temperature for overnight. Purification by column chromatography on silica gel using *n*-hexane–acetone (20:1) as eluted yield 24.0 mg (82%) of **10**. Colorless oil; ^{1}H NMR ($CDCl_3$): δ 1.71 (3H, s), 1.76 (3H, s), 3.31 (2H, d, J = 7.6 Hz), 3.79 (3H, s), 3.86 (3H, s), 5.29 (1H, t, J = 7.6 Hz), 6.30 (1H, d, J = 16.1 Hz), 6.83 (1H, d, J = 8.4 Hz), 7.32 (1H, d, J = 2.3 Hz), 7.35 (1H, dd, J = 8.4, 2.3 Hz), 7.64 (1H, d, J = 16.1 Hz); ^{13}C NMR ($CDCl_3$): δ 167.8, 159.2, 145.0, 133.1, 130.1, 128.9, 127.7, 126.7, 121.7, 114.8, 110.2, 55.4, 51.4,

28.2, 25.7, 17.7; DARTMS $[M+H]^+$ *m/z* 261; HRDARTMS $[M+H]^+$ *m/z*: 261.1488 (calcd for $C_{16}H_{21}O_3$: 261.1491).

4.2.3.2. 3-(4-Hydroxy-3-prenylphenyl)acrylic acid methyl ester (11)[34]. Compound **9** (91.0 mg, 0.39 mmol) was dissolved in 0.5 mL of methanol, then $TMSCH_2N_2$ (0.5 mL) was added and stirred at 0 °C for 30 min. Purification by column chromatography on silica gel using *n*-hexane–EtOAc (5:1) as eluted yield 63.7 mg (66%) of **11**. White powder; mp 86 °C (lit. 78–82 °C)[35]; ^{1}H NMR ($CDCl_3$): δ 1.78 (3H, s), 1.79 (3H, s), 3.36 (2H, d, J = 7.6 Hz), 3.80 (3H, s), 5.31 (1H, t, J = 7.6 Hz), 5.68 (1H, br s), 6.29 (1H, d, J = 16.1 Hz), 6.81 (1H, d, J = 8.4 Hz), 7.29 (1H, br s), 7.30 (1H, dd, J = 8.4, 2.3 Hz), 7.63 (1H, d, J = 16.1 Hz); ^{13}C NMR ($CDCl_3$): δ 168.3, 156.6, 145.3, 135.0, 134.9, 130.1, 127.7, 126.9, 121.2, 116.0, 114.6, 51.7, 29.2, 25.8, 17.8; DARTMS $[M+H]^+$ *m/z* 247; HRDARTMS $[M+H]^+$ *m/z*: 247.1330 (calcd for $C_{15}H_{19}O_3$: 247.1334).

4.2.3.3. 3-(4-Methoxy-3-prenylphenyl)acrylic acid (12)[34]. Compound **10** (47.9 mg, 0.18 mmol) was dissolved in 3.0 mL of THF, then KOH (25.0 mg, 0.45 mmol) and H_2O (50 μL) were added, and the solution was heated to reflux. After reaction completion (3 h) the mixture was brought to pH 4 by the addition of 2 N HCl and extracted three times with Et_2O and the combined organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure to afford **12** as white solid (44.6 mg, 99%). Mp 153–154 °C; ^{1}H NMR (acetone-d_6): δ 1.72 (6H, s), 3.32 (2H, d, J = 7.6 Hz), 3.89 (3H, s), 5.32 (1H, t, J = 7.6 Hz), 6.36 (1H, d, J = 16.1 Hz), 6.99 (1H, d, J = 8.4 Hz), 7.47 (1H, d, J = 2.3 Hz), 7.49 (1H, dd, J = 8.0, 2.3 Hz), 7.62 (1H, d, J = 16.1 Hz); ^{13}C NMR (acetone-d_6): δ 168.3, 160.2, 145.7, 133.0, 131.3, 129.8, 128.8, 127.7, 123.0, 116.2, 111.4, 55.9, 29.0, 25.9, 17.8; DARTMS $[M+H]^+$ *m/z* 247; HRDARTMS $[M+H]^+$ *m/z*: 247.1338 (calcd for $C_{15}H_{19}O_3$: 247.1334).

4.2.3.4. 4-Hydroxyphenyl-acrylic acid methyl ester (14)[36]. 4-Hydroxycinnamic acid (**13**, 300.0 mg, 1.83 mmol) was dissolved in 1.5 mL of methanol, catalytic amount of concd H_2SO_4 and 4 Å MS were added, and the solution was heated to reflux. After reaction completion (29 h) the solvent was evaporated in vacuo. The residue was extracted three times with EtOAc and the combined organic layer was washed with brine and satd $NaHCO_3$, dried over anhydrous Na_2SO_4, and then evaporated in vacuo to afford **14** as a white solid (236.0 mg, 72%). Mp 122–125 °C (lit. 124–130 °C)[37]; ^{1}H NMR (acetone-d_6): δ 3.72 (3H, s), 6.35 (1H, d, J = 16.1 Hz), 6.90 (2H, d, J = 8.4 Hz), 7.54 (2H, d, J = 8.4 Hz), 7.61 (1H, d, J = 16.1 Hz); ^{13}C NMR (acetone-d_6): δ 167.8, 160.5, 145.3, 130.8, 126.9, 116.6, 115.2, 51.5; DARTMS $[M+H]^+$ *m/z* 179; HRDARTMS $[M+H]^+$ *m/z*: 179.0701 (calcd for $C_{10}H_{11}O_3$: 179.0708).

4.2.3.5. 3-(4-Allyloxyphenyl)acrylic acid methyl ester (15)[38]. Compound **14** (99.5 mg, 0.56 mmol) was dissolved in 1.5 mL of acetone, 108.1 mg (0.78 mmol) K_2CO_3 and 0.10 mL (1.17 mmol) allyl bromide were added, and the solution was heated to reflux. After reaction completion (1.5 h) the solvent was evaporated in vacuo. The residue was extracted three times with $CHCl_3$ and the combined organic layer was washed with brine, dried over anhydrous Na_2SO_4, and evaporated in vacuo. The residue was recrystallized (*n*-hexane-EtOAc) to afford **15** as a white solid (112.1 mg, 92%). Mp 53–56 °C (lit. 65.5 °C)[38]; ^{1}H NMR ($CDCl_3$): δ 3.79 (3H, s), 4.57 (2H, d, J = 5.3, 1.4 Hz), 5.31 (1H, dq, J = 10.5, 1.5 Hz), 5.42 (1H, dq, J = 17.1, 1.5 Hz), 6.00–6.10 (1H, m), 6.31 (1H, d, J = 15.9 Hz), 6.91 (2H, d, J = 8.7 Hz), 7.47 (2H, d, J = 8.7 Hz), 7.65 (1H, d, J = 15.9 Hz); ^{13}C NMR ($CDCl_3$): δ 167.8, 160.4, 144.5, 132.8, 129.7, 127.3, 118.0, 115.4, 115.1, 68.8, 51.6, 29.7; DARTMS $[M+H]^+$ *m/z* 219; HRDARTMS $[M+H]^+$ *m/z*: 219.0997 (calcd for $C_{13}H_{15}O_3$: 219.1021).

4.2.3.6. 3-(4-Hydroxy-3-allylphenyl)acrylic acid methyl ester (16)[39]. Compound **15** (54.2 mg, 0.25 mmol) was dissolved in 3.0 mL of *N*,*N*-diethylaniline, and the solution was heated to reflux. After reaction completion (5 h) the mixture was brought to pH 4 by the addition of 2 N HCl and extracted three times with Et_2O and the combined organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure. The residue was purified by prep. TLC (*n*-hexane–EtOAc = 5:1) to afford **16** as a brown oil (35.3 mg, 65%). ^{1}H NMR ($CDCl_3$): δ 3.42 (2H, d, J = 6.1 Hz), 3.80 (3H, s), 5.17 (1H, dd, J = 7.6, 1.5 Hz), 5.20 (1H, t-like, J = 1.5 Hz), 5.52 (1H, s), 5.97–6.00 (1H, m), 6.31 (1H, d, J = 16.1 Hz), 6.83 (1H, d, J = 7.6 Hz), 7.31 (1H, br s), 7.32 (1H, dd, J = 7.6, 3.1 Hz), 7.63 (1H, d, J = 16.1 Hz); ^{13}C NMR ($CDCl_3$): δ 168.2, 156.4, 145.0, 135.8, 130.5, 128.2, 127.1, 126.2, 116.9, 116.1, 114.9, 51.7, 34.7; DARTMS $[M+H]^+$ *m/z* 219; HRDARTMS $[M+H]^+$ *m/z*: 219.0997 (calcd for $C_{13}H_{15}O_3$: 219.1021).

4.2.3.7. 3-(4-Hydroxy-3-allylphenyl)acrylic acid (17)[38]. Compound **16** (23.4 mg, 0.11 mmol) was dissolved in 3.0 mL of THF, then KOH (20.0 mg, 0.36 mmol) and H_2O (50 μL) were added, and the solution was heated to reflux. After reaction completion (3 h) the mixture was brought to pH 4 by the addition of 2 N HCl and extracted three times with Et_2O and the combined organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure. The residue was recrystallized ($CHCl_3$) to afford **17** as a white solid (14.7 mg, 67%). Mp 178–180 °C; ^{1}H NMR (acetone-d_6): δ 3.41 (2H, d, J = 6.8 Hz), 5.01–5.05 (1H, m), 5.10 (1H, dq, J = 17.1, 1.8 Hz), 5.98–6.09 (1H, m), 6.33 (1H, d, J = 15.9 Hz), 6.91 (1H, d, J = 8.2 Hz), 7.39 (1H, dd, J = 8.2, 2.4 Hz), 7.45 (1H, d, J = 2.4 Hz), 7.60 (1H, d, J = 15.9 Hz); ^{13}C NMR (acetone-d_6): δ 170.9, 158.0, 145.7, 137.5, 130.9, 128.7, 128.0, 127.1, 116.1, 115.8, 115.6, 34.7; DARTMS $[M+H]^+$ *m/z* 205; HRDARTMS $[M+H]^+$ *m/z*: 205.0881 (calcd for $C_{12}H_{13}O_3$: 205.0865).

4.2.3.8. 3-(3,5-Di-*tert*-butylphenyl-4-hydroxy)acrylic acid (18)[40]. 3,5-Di-*tert*-butylphenyl-4-hydroxybenzaldehyde (300.0 mg, 1.28 mmol) and malonic acid (199.6 mg, 1.92 mmol) were dissolved in 6.0 mL of pyridine, catalytic amount of piperidine was added and stirred at 40 °C for overnight. After reaction completion the mixture was brought to pH 4 by the addition of 2 N HCl and extracted three times with EtOAc and the combined organic layer was washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure. The residue was purified by recrystallization ($CHCl_3$/*n*-hexane) to afford **18** as a white solid (130.9 mg, 37%). Mp 203–204 °C (lit. 206–208 °C)[40]; ^{1}H NMR ($CDCl_3$): δ 1.46 (18H, s), 5.56 (1H, br s), 6.32 (1H, d, J = 16.1 Hz), 7.40 (2H, s), 7.76 (1H, d, J = 16.1 Hz); ^{13}C NMR ($CDCl_3$): δ 172.8, 156.6, 148.2, 136.4, 125.8, 125.5, 113.7, 34.3, 30.1; DARTMS $[M+H]^+$ *m/z* 277; HRDARTMS $[M+H]^+$ *m/z*: 277.1815 (calcd for $C_{17}H_{25}O_3$: 277.1804).

4.3. Biological studies

4.3.1. Preparation of test compounds

Compounds were prepared as stock solutions in DMSO and stored in aliquots at −20 °C. The final concentration of DMSO was 1.0% (v/v) in the biological assays unless otherwise noted.

4.3.2. Cell culture, transfection of the reporter gene, and cloning of stable transformants

HEK293 cell lines were maintained in Eagle's minimum essential medium with 1% (v/v) nonessential amino acids (GIBCO), supplemented with 10% (v/v) fetal bovine serum (FBS; Nichirei), 50 units/mL penicillin, 50 μg/mL streptomycin, and 50 μg/mL kanamycin (Meiji). Reporter plasmid p2.1 contained a 68-bp hypoxia response element from the *ENO1* gene inserted upstream of an

SV40 promoter in the luciferase reporter plasmid pGL2-promoter (Promega).[20] The stable transformants of the HEK293 cells for the HIF-1-dependent promoter assay were established by the transfection of p2.1 along with a pcDNA 3.1 empty vector containing a neomycin resistant gene using TransFection™ lipid reagent (Bio-Rad), followed by selection with Geneticin (G418) sulfate.

4.3.3. Hypoxia treatment

Hypoxic incubation (1% O_2) was performed in an air-tight modular incubator chamber (Billups-Rothenberg, Inc.) flushed with a gas mixture containing 1% O_2, 94% N_2 and 5% CO_2. The chamber was placed in a 37 °C incubator. For normoxic conditions (20% O_2), cells were incubated in a standard tissue culture incubator in 95% air and 5% CO_2.

4.3.4. Cell-based luciferase reporter assay

HEK293 clone cells were plated into 24-well plates (TPP Techno Plastic Products AG) at an initial density of 8×10^4 cells/well and cultured for 24 h with regular growth medium. After 24 h, the medium was replaced with a fresh medium containing the test compounds. After 1 h, they were incubated for 24 h under normoxic or hypoxic conditions. The assay was conducted according to the luciferase assay kit instructions (Roche). The activity was measured using a luminometer (Berthold Detection Systems, Sirius.). The final concentration of DMSO is 0.5% (v/v).

4.3.5. MTT assay

HCT116 cells were plated at a density of 6.0×10^3 cells/well (96-well plate, TPP Techno Plastic Products AG) in 100 μL of McCoy's 5A medium with 10% FBS and antibiotics, incubated for 24 h, and exposed to the test compounds under normoxic or hypoxic conditions for 24 h. The MTT reagent (0.5 mg/mL, Sigma-Aldrich Japan) was added to the media. After 4 h incubation at 37 °C, the media were removed, and the cells were lysed with DMSO. Absorbance at 570 nm was measured on a Multiskan JX plate reader (Thermo Fisher Scientific).

4.3.6. Clonogenic assay

HCT116 cells were plated at a density of 3.0×10^2 cells in a φ6 dish (TPP Techno Plastic Products AG) and treated with the test compounds for 24 h under normoxic or hypoxic conditions. On day 7 after colonies were formed and washed with PBS, the cells were fixed in methanol for 30 min at room temperature, stained with Giemsa's solution over 3 h, and washed with water. These colonies were then counted.

4.3.7. Immunoblot assays

HCT116 cells were plated at a density of 4×10^6 cells/well (φ10 dish, TPP Techno Plastic Products AG) in 8 mL of McCoy's 5A medium with 10% FBS and antibiotics described above, incubated for 24 h, and exposed to compounds under normoxic or hypoxic conditions for 4 h. Treated cells were rinsed twice with ice-cold PBS and lysed in appropriate extraction buffer [50 mM Tris–HCl, pH 8; 150 mM sodium chloride; 0.1% (v/v) sodium dodecyl sulfate (SDS); 1% (v/v) Tergitol solution; 5 mM ethylenediaminetetraacetic acid; 0.5% (w/v) sodium deoxycholate; 0.1 mM dithiothreitol; 1 mM phenylmethylsulfonyl fluoride; 1 mM $NaVO_4$; 10 mM sodium fluoride; and one-half of tablet Complete® (Roche)] for 30 min on ice. The extracts were centrifuged for 30 min at 15000 rpm. The protein concentrations were determined using a micro BCA assay kit (Pierce). Each sample was loaded at 120 μg protein/lane on 7% SDS–polyacrylamide gel. After electrophoresis, proteins were electrotransferred to nitrocellulose membranes. The membranes were blocked for 1 h at room temperature in Tris-buffered saline plus 0.1% Tween 20 containing 5% skim milk (Wako), and then incubated for 1.5 h at room temperature with a 1:500 dilution of one of the mouse monoclonal antibodies against human HIF-1α (Novus Biologicals Inc.) or goat polyclonal anti-human β-actin (1:3000 dilution, Santa Cruz). After washing, the membranes were incubated for 1 h at room temperature with 1:1000 or 1:3000 dilutions of appropriate horseradish peroxidase-labeled secondary antibody (Sigma), and bound antibody was visualized and quantified by chemiluminescence detection using a LAS 3000 mini imager and Multi Gauge software (Science Lab., version 2005, version 3.0; Fuji Photo).

4.3.8. RNA extraction and real-time RT-PCR

HCT116 cells were plated at a density of 1×10^6 cells/well (φ10 dish) in 8 mL of McCoy's 5A medium with 10% FBS and antibiotics, incubated for 24 h, and exposed to compounds under normoxic or hypoxic conditions for 24 h. Total RNA samples were extracted immediately following treatments with the Isogen (Nippongene). Synthesis of the first strand cDNAs was performed using ReverTra Ace® qPCR RT kit (TOYOBO) per the manufacturer's instructions. Quantitative real-time PCR was performed in triplicate using the Thermal Cycler Dice® Real Time system (TaKaRa Bio). Independent PCRs were performed using the same cDNA samples for the gene of interest and *18S rRNA* using SYBR® Premix Ex Taq (TaKaRa Bio). The following gene-specific pairs were used: *GLUT1*, 5′-ACTGCAA CGGCTTAGACTTCGAC-3′, 5′-ACAAACTAGGGACAATGGGTCTCT-3′; *VEGFA*, 5′-TGCTTCTGAGTTGCCCAGGA-3′, 5′-GATACAGGAGTGTGG TAACTTTGGT-3′; *HK2*, 5′-ATGCCTAGATGACTTCCGCACA-3′, 5′-ACA-TGGACCCACTCTAACAGGC-3′; and *18S rRNA*, 5′-CGTTGATCCTGCCAG-TAGC-3′, 5′-CAATACCAAGGAAACCAGCG-3′. The PCR conditions consisted of denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s, and annealing/extension at 60 °C for 30 s. Data generated from each PCR reaction were analyzed using Thermal Cycler Dice Real® Time System Software ver. 3.0 (TaKaRa Bio). The C_T value of *18S rRNA* was subtracted from that of the gene of interest to obtain a ΔC_T value. The C_T value of an arbitrary calibrator (e.g., untreated sample in the case of upregulated genes) was subtracted from the ΔC_T value to obtain a $\Delta\Delta C_T$ value. The gene expression level relative to the calibrator was expressed as $2^{-\Delta\Delta CT}$.

4.3.9. CAM assay in fertilized chicken eggs

The effect of the test compounds on angiogenesis was determined by CAM assay.[41] Briefly, fertilized chicken eggs were incubated at 37.5 °C in a humidified incubator with forced air circulation. Ovalbumin (3 mL) was removed from 3-day-old embryonated eggs. Thereafter, windows were opened for each egg, coated with caps, and eggs were incubated at 39.5 °C. On day 5, samples in saline solution with 2.0% DMSO and 1.0% methylcellulose were applied in the center of silicon rings (outer diameter: 5 mm; inner diameter: 3 mm; height: 1 mm) on the CAM, and the eggs were incubated at 39.5 °C for 2 days. The assay was scored and photographed on the 7th embryonic day. Saline solution with 2.0% DMSO and 1.0% methylcellulose was used as a vehicle. Ten eggs were used in total for each data point. Inhibition point was judged by estimation of area of the avascular zone. The inhibition ratios were calculated from the following formula; inhibition ratio (%) = [1 − (control point/drug point)] × 100.

4.4. Quantitative analysis by LC–ESI-MS

Quantitative chromatographic analysis of the Brazilian green propolis extract was performed using a HP 1100 Series and a HP LC/MSD (Agilent Technologies). A GL-Cart cartridge column ODS-80A and Waters Symmetry® C18 3.5 μm column (4.6 × 75 mm) were used. The mobile phase consisted of buffer solution in reservoir A (93.9% water, 0.8% acetic acid, 0.3% ammonium acetate, and 5% methanol) and acetonitrile in reservoir B. The elution was

performed using a linear gradient of 25–90% B over 90 min at a flow rate of 0.5 mL/min. The column was operated at a constant temperature of 40 °C. Detection was performed 254 and 280 nm using a diode array detector. MS analysis was performed in the negative mode on a HP 1100 MSD Series equipped with an ESI interface and LC/MSD Chemstation software (Agilent Technologies). Veratraldehyde was used as the internal standard.[32] Conventional ESI-MS data were recorded using a scan range of *m/z* 150–500. Active compounds were identified by retention time and molecular ion peak (see the MS data in the Supplementary data).

4.5. Statistical analysis

For the result from the luciferase assay (first screening) and real-time RT-PCR assays, statistical differences between cells treated with control and with compounds were evaluated by Student's *t*-test as indicated in the figures ($^{*}p$ <0.05, $^{**}p$ <0.01).

Dose-response results of the luciferase assay are presented as the mean ± SD of three independent experiments, with each treatment performed in triplicate. Statistical significance was determined by a post-hoc Dunnett's test for comparisons of treated samples versus controls, as indicated in the figures ($^{*}p$ <0.05).

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.07.060.

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