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Novel anticancer agents, kayeassamins C–I from the flower of *Kayea assamica* of Myanmar

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ABSTRACT

A $CHCl_3$-soluble fraction of 70% EtOH extract of the flower of *Kayea assamica* from Myanmar exhibited 100% preferential cytotoxicity (PC_{100}) against human pancreatic cancer PANC-1 cells under nutrient-deprived conditions at 1 μg/mL. Bioassay-guided fractionation and isolation afforded nine new coumarins, kayeassamins A (**8**), B (**9**), and C–I (**1**–**7**), together with nine known coumarins (**10**–**18**). The structures of these compounds were identified by extensive spectroscopic techniques as well as by comparison with published data. Absolute configuration at C-1′ of **1** was established as *S*-configuration by the modified Mosher method. All the isolates were evaluated for their in vitro preferential cytotoxicity using novel anti-austerity strategy. Among them, the novel coumarins, kayeassamins A (**8**), B (**9**), D (**2**), E (**3**), and G (**5**) exhibited the most potent preferential cytotoxicity (PC_{100} 1 μM) in a concentration- and time- dependent manner and induced apoptosis-like morphological changes of PANC-1 cells within 24 h of treatment. Based on the observed cytotoxicity, structure-activity relationships have been established.

1. Introduction

Pancreatic cancer is the fifth leading cause of cancer death with a median survival of <6 months and a relative 5-year survival rate of 5.5%. In 2005, it was estimated that 22,926 men and women died of pancreatic cancer in Japan.[1,2] It is most intrinsically resistant to the conventional anticancer drugs in clinical use such as 5-fluorouracil, taxol, doxorubicin, cisplatin and camptothecin, and is a major cause of treatment failure in pancreatic cancer. Therefore, the development of effective adjunct strategies is urgently necessary. Gemcitabine currently represents the standard chemotherapeutic drug for metastatic and advanced disease, but it only leads to a modest improvement in quality of life and survival.[3] Pancreatic tumors are known to have poor angiogenesis that leads to insufficient nutrition supply to the aggressively proliferating cancer cells. However, pancreatic cancer cells have inherent tolerance to survive under low nutrient conditions. Therefore, the ability of cancer cells to tolerate nutrient starvation (austerity) is regarded as another critical factor for tumor progression under hypovascular conditions. Hence, an agent that can retard the cancer cells' tolerance to nutrient starvation (anti-austerity agent) was considered as a novel target in anticancer drug discovery.[4–7]

In our continuing program to discover new anticancer agents based on a novel 'anti-austerity strategy',[8–12] we found that the $CHCl_3$-soluble fraction of 70% EtOH extract of the flower of *Kayea assamica* King & Prain (Clusiaceae) collected from Myanmar, showed potent cytotoxicity to PANC-1 cells preferentially in nutrient-deprived conditions at 1 μg/mL. *K. assamica* is a slow growing, tall, evergreen tree and blooms from early in October to May. It is locally known as '*Tharapi*' and has been used to reduce extreme hotness in the body, dizziness, dry skin, and fever. In India, the fruits of this species are used as a fish poison, and the aqueous extract of the stem bark is used as remedy for treating fevers. The pollen is used for sores, fistulas, fever, and malaria.[13] Previous investigation of this plant reported cytotoxic and antimalarial alkylated coumarins from the bark, root bark, and fruit.[14] In the present study, we carried out bioassay-guided fractionation and isolation to identify preferentially cytotoxic anticancer agents, which afforded nine novel coumarins, kayeassamins A–I, together with nine known ones. Among the new compounds, kayeassamins A (**8**) and B (**9**) were reported in our preliminary communication.[15] In this paper, we report the isolation and identification of seven novel anticancer agents, kayeassamins C–I (**1–7**), together with the preferential cytotoxic activity of the isolated compounds.

2. Results and discussions

2.1. Isolation and identification

The 70% EtOH extract of the flowers of *K. assamica* showed 100% preferential cytotoxicity (PC_{100}) against PANC-1 cancer cells under

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nutrient-deprived conditions at 1 μg/mL. Thus, it was separated into the $CHCl_3$-soluble and -insoluble fractions. Among them, only the $CHCl_3$-soluble fraction exhibited preferential cytotoxicity at 1 μg/mL. This was therefore subjected to a series of chromatographic separation which led to the isolation of nine new coumarins, kayeassamins C–I (**1**–**7**), A (**8**)[15] and B (**9**),[15] and nine known ones [mammea A/AA cyclo D (**10**),[16,17] mammea A/BC (**11**),[18] mammea B/AC (**12**),[19,20] mammea A/AC (**13**),[21,22] mammea A/AC cyclo D (**14**),[21,22] a mixture of theraphins B and C (**15** and **16**),[14] mammea B/AC cyclo F (**17**),[17,19] and deacetylmammea E/BA cyclo D (**18**)[23]]. Their structures were determined by means of extensive spectroscopic techniques and by comparison of their published data.

Kayeassamin C (**1**) was obtained as pale yellow oil with $[\alpha]^{23}_{D}$ −60.87°. The molecular formula of **1** was established to be $C_{27}H_{36}O_6$ by HREIMS. The IR spectrum exhibited the absorption of hydroxyl group (3480 cm^{-1}), α,β-unsaturated lactone (1720 cm^{-1}), and chelated acyl group (1610 cm^{-1}). The UV spectrum showed absorption maxima at 220 and 329 nm in EtOH, similar to those of 5,7-dihydroxy coumarins.[20] The ^{1}H NMR spectrum of **1** displayed signals of an oxygenated methine (δ_H 4.78, H-1′), three olefinic methines (δ_H 5.06, H-7‴; 5.22, H-2‴; 6.14, H-3), one alkyl methine (δ_H 3.78, H-2″), five methylenes (δ_H 1.44, 1.85, H_2-4″; 1.76, 1.94, H_2-2′; 1.99, H_2-5‴; 2.06, H_2-6‴; 3.42, H_2-1‴), two primary and one secondary methyls (δ_H 0.96, H_3-5″; 1.02, H_3-3′; 1.23, H_3-3″), three vinyl methyls (δ_H 1.57, H_3-10‴; 1.65, H_3-9‴; 1.81, H_3-4‴), and three hydroxyl protons (δ_H 14.27, 7-OH; 10.51, 5-OH; 4.18, 1′-OH) (Table 1). Meanwhile, ^{13}C NMR spectrum of **1** indicated 27 carbons, including those for a ketone carbonyl carbon, a lactone carbonyl carbon, six aromatics, one oxygenated methine, three olefinic methines, one alkylated methine, five methylenes, six methyls, and three quaternary olefinic carbons (Table 2). Furthermore, COSY and HMBC correlations (Fig. 1a) indicated the presence of a hydroxypropyl, a 2-methyl-1-oxobutyl, and a geranyl substituent. These data match well to those of surangin C, previously isolated from the *Mammea longifolia*[24] and the yellow batai (*Peltophorum dasyrachis*).[25] However, they differ from each other due to the presence of an additional hydroxyl proton at δ_H 10.51 (5-OH) in **1**, a characteristic of 5,7-dihydroxy-6-acylcoumarin.[20] A bathochromic shift observed after the addition of alkali in its UV spectrum (Table 4) further suggested the location of the acyl group to be at C-6.[20] Therefore, the 2-methyl-1-oxobutyl unit was fixed at C-6, which was confirmed by HMBC correlations of the chelated hydroxyl proton at δ_H 14.27 (7-OH) with C-6, C-7, and C-8. In addition, the HMBC correlations of H-1′ with C-3 and C-4a, of H-2′ with C-4, and of H-1‴ with C-7, C-8, and C-8a indicated the location of the hydroxypropyl group at C-4 and the geranyl unit at C-8. Based on this evidence, the planar structure of kayeassamin C (**1**) was established as 5,7-dihydroxy-4-(1-hydroxypropyl)-6-(2-methyl-1-oxobutyl)-8-(3,7-dimethyl-2,6-octadienyl)-2*H*-benzopyran-2-one. The absolute configuration at C-1′ of **1** was determined by the modified Mosher method.[26,27] The MTPA esters of **1** were obtained by treating **1** with (*R*)- and (*S*)-MTPA chloride and their ^{1}H NMR resonances were assigned based on COSY correlations.

The chemical shift differences ($\Delta_{SR} = \delta_S - \delta_R$) of the individual protons of **1a** and **1b** are shown in Figure 2. In the ^{1}H NMR spectrum of the (*S*)-MPTA ester (**1a**), H_2-2′ and H_3-3′ appeared shielded,

Table 2
^{13}C NMR data ($CDCl_3$, 100 MHz) for kayeassamins C–G (**1**–**5**)

Carbon	1	2	3	4	5
2	159.7	159.8	160.8	159.2	159.9
3	109.0	109.1	108.9	108.8	108.9
4	156.3	156.4	156.8	155.9	156.4
4a	100.7	100.6	100.6	100.3	100.7
5	156.2	156.2	156.2	155.4	156.2
6	104.2	104.3	103.9	103.8	104.3
7	166.3	166.5	166.7	165.9	166.6
8	113.8	113.9	114.8	113.5	114.2
8a	157.7	157.7	157.5	157.2	157.7
1′	77.5	77.7	78.4	77.4	77.9
2′	28.2	28.1	27.5	27.7	27.9
3′	10.5	10.5	10.5	10.1	10.5
1″	210.4	205.5	205.2	209.9	205.5
2″	47.1	53.7	46.8	46.6	53.7
3″	16.6	25.4	17.9[a]	16.2	25.4
4″	27.2	22.6[a]	13.8	26.8	22.6[a]
5″	11.7	22.6[a]		11.3	22.6[a]
1‴	21.9	21.9	21.9	22.3	22.0
2‴	121.2	121.2	121.4	120.9	121.4
3‴	137.4	137.3	133.0	133.2	133.5
4‴	16.3	16.3	25.8	25.4	25.8
5‴	39.8	39.8	17.9[a]	17.5	17.9
6‴	26.7	26.7			
7‴	124.2	124.1			
8‴	131.5	131.5			
9‴	25.7	25.7			
10‴	17.7	17.7			

[a] Overlapping resonances within the same column.

Table 1
^{1}H NMR data ($CDCl_3$, 400 MHz) for kayeassamins C–G (**1**–**5**), *J* values (in Hz) in Parentheses

Proton	1	2	3	4	5
3	6.14 s	6.09 s	6.01 s	6.14 s	6.03 s
1′	4.78 t (6.4)	4.75 t (7.8)	4.61 t (7.8)	4.78 t (7.3)	4.69 t (7.3)
2′	1.76 m; 1.94 m	1.78 m; 1.92 m	1.74 m; 1.98 m	1.76 m; 1.92 m	1.78 m; 1.97 m
3′	1.02 t (7.3)	1.02 t (7.6)	1.00[a] t (7.6)	1.03 t (7.3)	1.02 t (7.3)
2″	3.78 dd (6.6, 13.2)	2.99 dd (6.6, 15.7); 3.07 dd (6.6, 15.7)	3.00 m	3.82 dd (6.6, 12.9)	2.96 dd (6.6, 15.6) 3.05 dd (6.6, 15.6)
3″	1.23 d (6.6)	2.23 m	1.71 m	1.23 d (6.6)	2.21 m
4″	1.44 m; 1.85 m	1.00[a] d (4.5)	1.00[a] t (7.6)	1.45 m; 1.85 m	0.99[a] m
5″	0.96 t (7.3)	1.00[a] d (4.5)		0.96 t (7.3)	0.99[a] m
1‴	3.42 t (6.5)	3.40 t (7.3)	3.34 m	3.42 t (7.0)	3.39 t (7.0)
2‴	5.22 t (6.5)	5.20 t (7.3)	5.15 t (6.8)	5.22 t (7.0)	5.20 t (7.0)
4‴	1.81 s	1.81 s	1.67 s	1.71 s	1.70 s
5‴	1.99 m	1.99 m	1.79 s	1.81 s	1.80 s
6‴	2.06 m	2.04 m			
7‴	5.06 t (6.8)	5.06 t (7.1)			
9‴	1.65 s	1.64 s			
10‴	1.57 s	1.57 s			
1′-OH	4.18 br s		5.38 br s	3.71 br s	
5-OH	10.51 br s	10.58 br s	11.41 br s	10.39 br s	10.88 br s
7-OH	14.27 s	14.42 s	14.32 s	14.28 s	14.39 s

[a] Overlapping resonances within the same column.

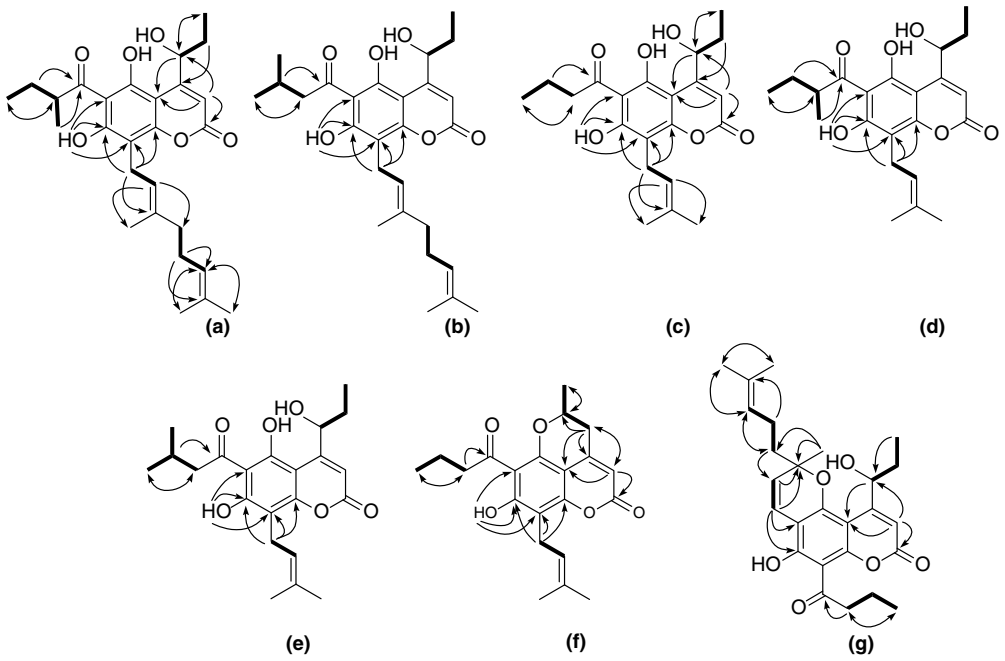
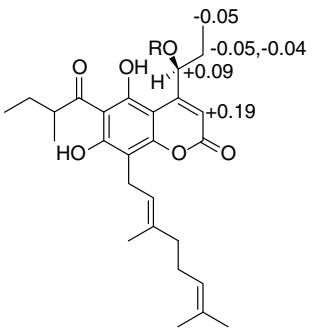


Figure 1. COSY (bold lines) and HMBC ($^1H \rightarrow {}^{13}C$) (arrows) correlations in **1**(a), **2**(b), **3**(c), **4**(d), **5**(e), **6**(f), and **7** (g).

whereas H-3 was deshielded, in comparison to the corresponding signals of (*R*)-MTPA ester (**1b**). Thus, H_2-2′ and H_3-3′ of **1a** were more affected by the phenyl ring of the MTPA part that indicated the absolute configuration of C-1′ in **1** to be *S*.

Kayeassamin D (**2**) was isolated as a pale yellow oil with $[\alpha]^{23}_D$ −47.90°. The IR and UV data of **2** (Table 4) showed similar patterns to those of **1**, indicating **2** to be a 5,7-dihydroxy-6-acylcoumarin. HREIMS of **2** also showed the same molecular formula $C_{27}H_{36}O_6$ as **1**. Extensive analyses of ^1H and ^{13}C NMR data (Tables 1 and 2) and COSY and HMBC correlations (Fig. 1b) indicated that **2** differed only on a 3-methyl-1-oxobutyl side chain at C-6. Kayeassamin D (**2**) showed negative optical rotation, similar to that of **1**, suggesting the absolute configuration at C-1′ to be *S*. Therefore, the structure of kayeassamin D (**2**) was established as (−)-5,7-dihydroxy-4-(1*S*-hydroxypropyl)-6-(3-methyl-1-oxobutyl)-8-(3,7-dimethyl-2,6-octadienyl)-2*H*-benzopyran-2-one.

Kayeassamin E (**3**) was isolated as a pale yellow oil with $[\alpha]^{23}_D$ −9.79°. Its molecular formula $C_{21}H_{26}O_6$ was established by HREIMS. The ^1H and ^{13}C NMR data of **3** (Tables 1 and 2) resembled those of theraphin A,[14] an isolate from the same species, and indicated the presence of a hydroxypropyl, an oxobutyl, and an isoprenyl substituent. However, besides the signal due to a chelated hydroxyl proton at δ_H 14.32 (7-OH), the ^1H NMR spectrum of **3** showed an additional signal ascribable to a hydroxyl proton at δ_H 11.41 (5-OH). Therefore, kayeassamin E (**3**) was assumed to be 6-acylcoumarin, a regioisomer of theraphin A. This conclusion was further supported by the bathochromic shift from 328 to 380 nm after addition of alkali in UV spectra (Table 4). Kayeassamin E (**3**) showed negative optical rotation as in theraphins A–D[14] and siamenols A–D.[28] Therefore the absolute configuration at C-1′ in **3** should be *S*. Accordingly, the structure of **3** was assigned as (−)-5,7-dihydroxy-4-(1*S*-hydroxypropyl)-6-(1-oxobutyl)-8-(3-methylbut-2-enyl)-2*H*-benzopyran-2-one.



1a R = (*S*)-MTPA
1b R = (*R*)-MTPAa

Figure 2. Difference in the Δ_{SR} ($\delta_S - \delta_R$) values for the (*S*)- and (*R*)-MTPA esters of **1** in $CDCl_3$.

Kayeassamins F (**4**) and G (**5**) were obtained as pale yellow oils with $[\alpha]^{23}_D$ −9.49° and −33.23°, respectively. They showed the same molecular formula $C_{22}H_{28}O_6$ in HREIMS. Moreover, they exhibited similar UV patterns as **3** (Table 4), ascribable to 6-acylcoumarin. The ^1H and ^{13}C NMR data (Tables 1 and 2) were similar to those of theraphins B and C.[14] However, the appearance of an additional hydroxyl broad singlet (δ_H 10.39 in **4**, δ_H 10.88 in **5**) in the ^1H NMR spectra of **4** and **5** suggested that they were regioisomers of theraphins B and C, respectively. The absolute configuration at C-1′ of **4** and **5** was also assumed to be *S* based on their negative optical activity. Therefore, the structures of **4** and **5** were concluded as shown in Chart 1.[29,30]

Kayeassamin H (**6**) was isolated as a pale yellow oil with $[\alpha]^{23}_D$ −31.95°. Its molecular formula $C_{21}H_{24}O_5$ was established by HREIMS. The ^1H and ^{13}C NMR data of **6** closely resembled those of theraphin A[14] and showed the signals of an oxobutyl, and an isoprenyl substituent together with a singlet olefinic proton ascribable to H-3 at δ_H 5.88 and a chelated hydroxyl proton at δ_H 14.20 (8-OH). However, significant differences were observed in the ^1H NMR spectrum of **6** for the signals having been assigned to the hydroxypropyl unit at C-4 in theraphin A. The COSY and HMQC spectra led to the assignment of the partial structure C(1′)H_3–C(5)H(O)–C(4)H_2 (Fig. 1f) in **6**, which was confirmed by the HMBC spectrum (Fig. 1f). This, together with the consideration of its molecular formula, indicated that **6** should be a pyranocoumarin, similar to those reported from *Mammea siamensis*.[31]

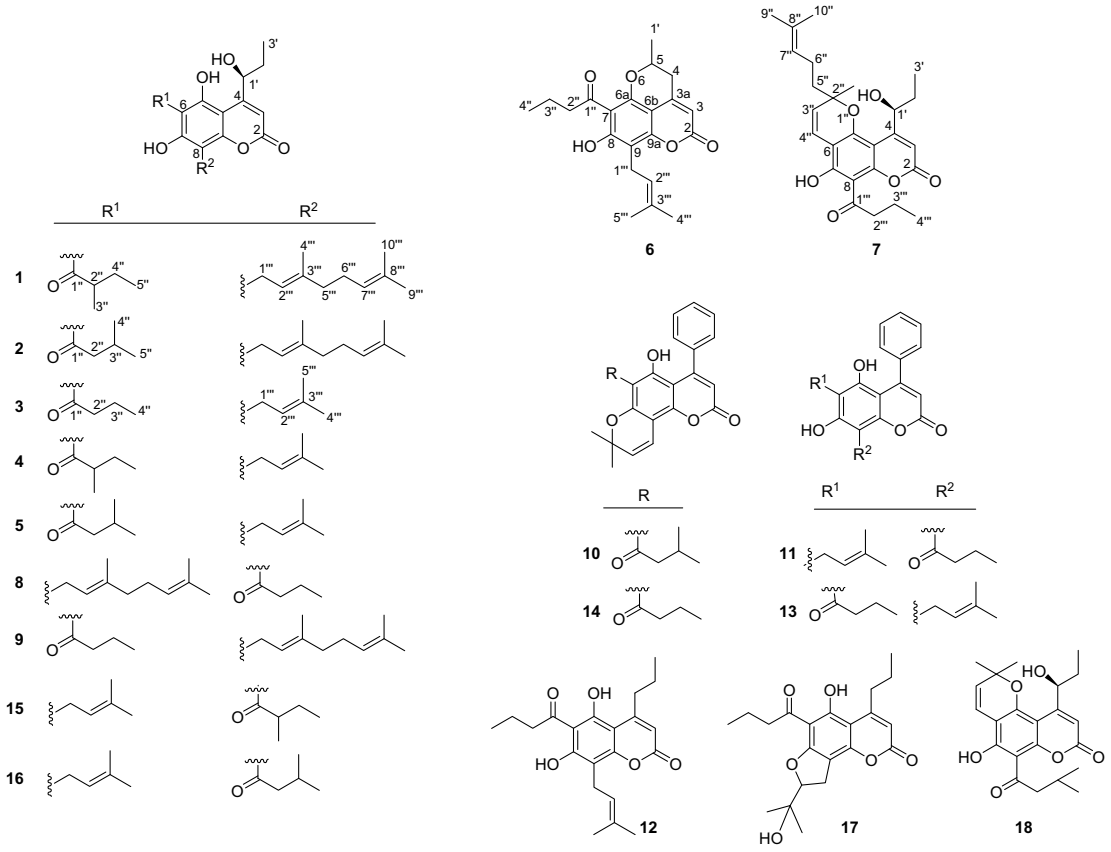


Chart 1. Structures of isolated coumarins (**1–18**) from the flower of *K. assamica*.

However, the HMBC and UV spectral data[32] indicated the acyl group to be at C-7. Therefore, the structure of kayeassamin H (**6**) was concluded as 8-hydroxy-5-methyl-7-(1-oxobutyl)-9-(3-methylbut-2-enyl)-4,5-dihydropyrano [4,3,2-*de*]chromen-2-one.

Kayeassamin I (**7**) was isolated as a pale yellow oil and its molecular formula was deduced to be $C_{26}H_{32}O_6$ by HREIMS. Its UV spectrum showed absorption at 320 nm (Table 4), suggesting it to be a 7-hydroxycoumarin derivative.[33] The ^{1}H NMR data of **7** (Table 3) displayed an oxygenated methine (δ_H 5.43, H-1′), four olefinic methines (δ_H 5.07, H-7″; 5.54, H-3″; 6.61, H-3; 6.79, H-4″), five methylenes (δ_H 1.50, 1.97, H_2-2′; 1.79, H_2-3‴; 1.90, H_2-5″; 2.09, H_2-6″; 3.27, H_2-2‴), five methyls (δ_H 1.05, H_3-4‴; 1.13, H_3-3′; 1.51, 2″-Me; 1.55, H_3-10″; 1.64, H_3-9″), and a hydrogen-bonded hydroxyl proton (δ_H 14.48, 7-OH). On the other hand, the ^{13}C NMR spectrum of **7** exhibited 26 carbon signals including those of a ketone carbonyl carbon, a lactone carbonyl carbon, six aromatics, an oxygenated methine, an oxygenated quaternary carbon, five methylenes, five methyls, four olefinic methines, and two quaternary olefinic carbons. These data closely resembled those of deacetylmammea E/BA cyclo D (**18**),[23] a known isolate in the present work, except for the presence of additional signals due to a methyl (δ_H 1.51), an olefinic methine (δ_H 5.07), two methylenes (δ_H 1.90, 2.09) and 1-oxobutyl unit. The HMBC correlations of H-3″, H-4″, H-5″, and 2″-Me with C-2″ and of H-6″ with C-7″ and C-8″ indicated the presence of a 2-methyl-2-(4-methylpent-3-enyl)-2*H*-pyran ring in **7** instead of the 2,2-dimethylpyran ring in **18**. Thus, the structure of kayeassamin I (**7**) was concluded as 7-hydroxy-2″-methyl-2″-(4-methylpent-3-enyl)-4-(1-hydroxypropyl)-8-(1-oxobutyl)-2*H*, 8*H*-benzo[1,2-*b*:3,4-*b*′]dipyran-2-one. Compound **7** exhibited negative optical rotation, similar to that of **18**,[23] and therefore the absolute configuration at C-1′ was assumed to be *S*.

2.2. In vitro preferential cytotoxicity

The effect of isolated coumarins on PANC-1 cell viability under nutrient-deprived conditions was determined in vitro and their preferential cytotoxicity (PC_{100}) is summarized in Table 5. Among the compounds tested, kayeassamins A (**8**), B (**9**), D (**2**), E (**3**), and G (**5**) were found to be the most potent with PC_{100} of 1 μM, which was comparable to that of the positive control, arctigenin.[8] The order of potency of the other isolates was **4**, **12** (2 μM) > **1** (4 μM) > **11** (8 μM) > **13** (16 μM) > **10**, **18** (32 μM) > **6**, **7**, **15–17** (64 μM) > **14** (> 256 μM). Upon careful inspection of the structure and activity, compounds possessing an isoprenyl or a geranyl substituent at C-8 and a hydroxypropyl substituent at C-4 showed the most potent activity. On C-6, presence of an oxobutyl or a 3-methyl-1-oxobutyl group was found to be favored than a 2-methyl-1-oxobutyl group (e.g., **2**, **9** > **1**; **3**, **5** > **4**). Interchange of geranyl and oxobutyl units at C-8 and C-6 did not alter the activity in the presence of a hydroxypropyl unit at C-4 (**8**≈**9**). However interchange of isoprenyl and any acyl groups between C-6 and C-8 led to a dramatic reduction in activity even in the presence of a hydroxypropyl group at C-4 (**15** << **4**; **16** << **5**). Replacement of the hydroxypropyl group at C-4 by a phenyl group led to significant decrease in activity (**13** < **3**). Similarly, presence of any additional cyclic ring in the coumarin nucleus led to the dramatic loss of activity (**3** >> **6**; **8** >> **7**; **12** >> **17**; **13** >> **14**).

Treatment by kayeassamins D (**2**), E (**3**), and G (**5**) at 1 μM triggered apoptosis-like morphological change in PANC-1 cells within 24 h (Fig. 4). The dying cells severed attachments to other cells and rounded up with clearly visible blebs. Furthermore, **2**, **3** and **5** showed preferential cytotoxicity in a concentration- and time-dependent manner (Fig. 3). Complete preferential cell death was observed within 12 h of treatment with **2**, and within 24 h of treat-

Table 3
^{1}H and ^{13}C NMR data for kayeassamins H (**6**) and I (**7**), *J* values (in Hz) in parentheses

Position	**6**		Position	**7**	
	δ_H	δ_C		δ_H	δ_C
2		160.3	2		159.6
3	5.88 s	106.3	3	6.61 s	107.0
3a		148.2	4		160.6
4	2.84 m	34.6	4a		101.0
5	4.41 m	73.8	5		155.9
6a		155.9	6		105.8
6b		99.4	7		162.9
7		110.5	8		104.5
8		165.8	8a		157.3
9		106.7	1′	5.43 d (8.1)	71.8
9a		155.7	2′	1.50 m; 1.97 m	30.7
1′	1.59 d (6.4)	20.7	3′	1.13 t (7.4)	10.2
1″		206.7	2″		83.0
2″	3.06 t (6.9)	46.8	3″	5.54 d (10.0)	124.9
3″	1.72 m	18.1	4″	6.79 d (10.0)	116.5
4″	1.01 t (7.5)	13.9	5″	1.90 m	41.6
1‴	3.44 d (7.3)	21.2	6″	2.09 m	23.0
2‴	5.23 t (7.3)	121.1	7″	5.07 t (7.1)	123.1
3‴		132.8	8″		132.6
4‴	1.67 s	25.8	9″	1.64 s	25.6
5‴	1.83 s	18.0	10″	1.55 s	17.7
8-OH	14.20 s		1‴		206.4
			2‴	3.27 t (7.1)	46.7
			3‴	1.79 m	18.1
			4‴	1.05 m	13.8
			2″-Me	1.51 s	27.3
			7-OH	14.48 br s	

Table 4
UV data of kayeassamins C–I (**1**–**7**)

Compound	λ_{max} nm (log ε)	
	In EtOH	In 0.1 N KOH in EtOH
1	220 (4.85), 329 (4.05)	211 (4.85), 380 (3.67)
2	207 (3.66), 329 (3.87)	211 (4.85), 380 (3.49)
3	223 (4.23), 328 (4.42)	213 (4.80), 380 (3.92)
4	224 (4.62), 328 (4.82)	213 (4.84), 380 (4.26)
5	224 (4.62), 328 (4.80)	213 (4.84), 380 (4.20)
6	207 (4.40), 246 (3.80), 281 (4.60)	212 (4.78), 323 (3.85), 391 (4.29)
7	220 (4.20), 271 (4.43), 320 (4.27)	212 (4.86), 383 (4.24)

Table 5
Preferential cytotoxicity of compounds (**118**) on human pancreatic PANC-1 cancer cells in nutrient-deprived conditions

Compound	PC_{100} [μM]
1	4
2, **3**, **5**, **8**, **9**	1
4, **12**	2
6, **7**, **15** + **16**, **17**	64
10, **18**	32
11	8
13	16
14	> 256
Arctigenin*	1

* Positive control.

ment with **3** and **5**, at 1 μM. This observation indicated that the geranyl moiety at C-8 might be an important feature for enhancement of activity. The sensitivities of the tested compounds were further increased when treated at the concentrations of 4 or 16 μM; more than 50% cells were killed within 3 h of exposure and the preferential cytotoxicity was observed within 9 h exposure. These observations indicate that kayeassamins D (**2**), E (**3**) and G (**5**) are potent anticancer agents, and might be useful for the treatment of pancreatic cancer in real clinical situations.

3. Conclusion

The $CHCl_3$-soluble fraction of the flower of *K. assamica* from Myanmar exhibited 100% preferential cytotoxicity (PC_{100}) at 1 μg/mL against the human pancreatic PANC-1 cancer cell line under nutrient-deprived medium (NDM). Bioassay-guided fraction and isolation led to the isolation of novel coumarins, kayeassamins A–I, together with nine known ones. All of the isolated compounds were evaluated for their preferential cytotoxicity against PANC-1 cells under NDM. Among them, five novel coumarins, kayeassamins A (**8**), B (**9**), D (**2**), E (**3**), and G (**5**), displayed the potent activity (PC_{100}, 1 μM). The structure–activity relationship of all the isolated coumarins demonstrated that a hydroxypropyl substituent at C-4, an oxobutyl or a 3-methyl-1-oxobutyl moiety at C-6, and a geranyl group at C-8 in 5,7-dihydroxycoumarin are important structural features in exhibiting the preferential cytotoxicity. Kayeassamins D (**2**), E (**3**), and G (**5**) induced apoptosis-like morphological changes in PANC-1 cells and exhibited preferential cytotoxicity in a concentration- and time-dependent manner.

4. Experimental

4.1. General experimental procedures

Optical rotations were recorded on a JASCO DIP-140 digital polarimeter. IR spectra were measured with a Shimadzu IR-408 spectrophotometer in $CHCl_3$. UV spectra were obtained using a Shimadzu UV-160A UV–visible recording spectrophotometer. NMR spectra were taken on a JEOL JNM-LA400 spectrometer with tetramethylsilane (TMS) as an internal standard, and chemical shifts are expressed in δ values. EIMS and HREIMS measurements were carried out on a JEOL JMS-700T spectrometer. Column chromatography was performed with normal-phase silica gel (Silica Gel 60N, Spherical, neutral, 40–50 μm, Kanto Chemical Co., Inc.) and reversed-phase silica gel (Cosmosil 75C_{18}-OPN, Nacalai Tesque Inc.). Analytical and preparative TLC were carried out on precoated silica gel 60F_{254} and RP-18F_{254} plates (Merck, 0.25 or 0.50 mm thickness). HPLC was performed with a Supelco Discovery C-18 column (25 cm × 10 mm, 5 μm) using Tosoh UV-8000 detector.

4.2. Plant material

Flowers of *Kayea assamica* King & Prain were collected from Taunggyi, Shan State, Myanmar in November, 2004. The plant was identified by Associate Professor Tin Maung Ohn (Department of Botany, University of Yangon, Myanmar). A voucher specimen (TMPW 25183) was deposited at the Museum for Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, University of Toyama, Japan.

4.3. Extraction and isolation

The flowers of *K. assamica* (1 kg) were extracted with 70% EtOH under sonication (2 L, 3× 90 min) at room temperature and the solvent was evaporated under reduced pressure to give 40 g of extract. Sonication of this extract with $CHCl_3$ (100 mL, 3× 90 min) afforded 14 g and 25 g each of $CHCl_3$-soluble and -insoluble fractions.

The $CHCl_3$-soluble fraction (14 g) was chromatographed over silica gel using increasing polarity of EtOAc in *n*-hexane to afford seven fractions [fr. 1: EtOAc/*n*-hexane (20:80) eluate, 4.88 g; fr. 2: EtOAc/*n*-hexane (30:70) eluate, 2.87 g; fr. 3: EtOAc/*n*-hexane (40:60) eluate, 2.34 g; fr. 4: EtOAc/*n*-hexane (50:50) eluate,

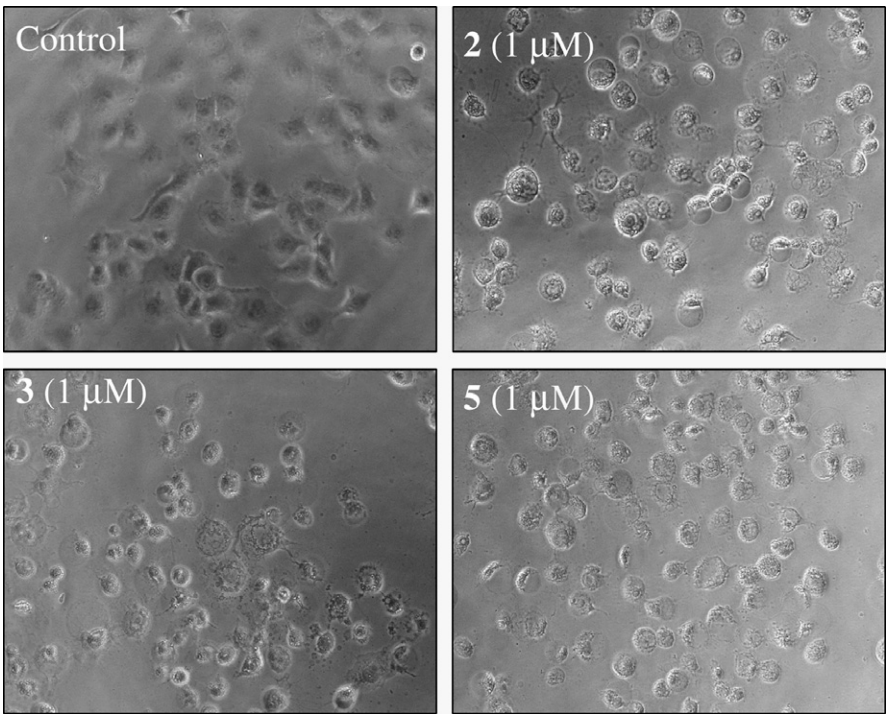


Figure 4. Morphological change of human pancreatic cancer PANC-1 cells under nutrient-deprived medium after 24 h exposure with 1 μM of **2**, **3** or **5**.

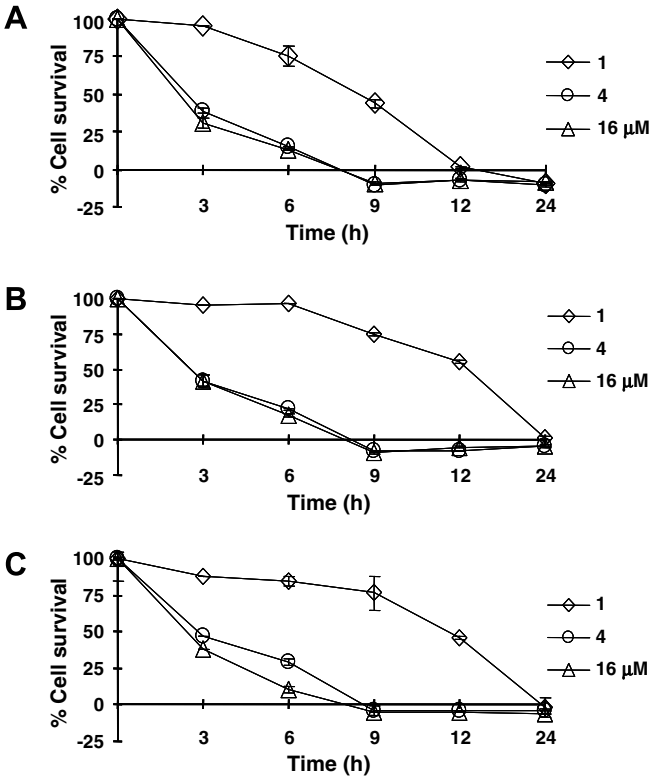


Figure 3. Survival of human pancreatic cancer PANC-1 cells under nutrient-deprived conditions within 024 h by 116 μM of **2** (A), **3** (B) and **5** (C). Data are means ± SEM, $n = 3$.

0.49 g; fr. 5: EtOAc/*n*-hexane (60:40) eluate, 0.63 g; fr. 6: EtOAc/*n*-hexane (80:20) eluate, 0.26 g; fr. 7: EtOAc eluate, 2.16 g]. Addition of MeOH to fr. 1 (4.88 g) crystallized mammea A/AA cyclo D (**10**, 4 g).

Si gel column chromatography (CC) on fr. 2 using CH_2Cl_2/*n*-hexane (2:1), CH_2Cl_2, and CH_2Cl_2/MeOH (40:1) afforded three subfractions [fr. 2-1: CH_2Cl_2/*n*-hexane (2:1) eluate, 671 mg; fr. 2-2: CH_2Cl_2 eluate, 188 mg; fr. 2-3: CH_2Cl_2/MeOH (40:1) eluate, 1.17 g]. Si gel CC of subfraction 2-1 (671 mg) with *n*-hexane/Me_2CO to give four fractions [fr. 2-1-1: *n*-hexane/Me_2CO (25:1) eluate, 125 mg; fr. 2-1-2: *n*-hexane/Me_2CO (15:1) eluate, 120 mg; fr. 2-1-3: *n*-hexane/Me_2CO (7:1) eluate, 54 mg; fr. 2-1-4: *n*-hexane/Me_2CO (3:1) eluate, 225 mg]. Subfraction 2-1-1 was subjected to normal-phase preparative TLC with benzene/Me_2CO (40:1) to give mammea A/BC (**11**, 46 mg) and mammea B/AC (**12**, 22 mg). Mammea A/AC (**13**, 60 mg) and mammea A/AC cyclo D (**14**, 40 mg) were obtained from subfractions 2-1-2 and 2-1-3 by normal-phase pTLC with benzene/Me_2CO (20:1).

Fraction 3 (2.34 g) was rechromatographed on reversed-phase Si gel with MeCN/H_2O to afford three subfractions [fr. 3-1: MeCN/H_2O (7:3) eluate, 524 mg; fr. 3-2: MeCN/H_2O (4:1) eluate, 270 mg; fr. 3-3: MeCN, 687 mg]. Si gel CC of fr. 3-1 using *n*-hexane/Me_2CO to give two subfractions [fr. 3-1-1: *n*-hexane/Me_2CO (20:1) eluate, 306 mg; fr. 3-1-2: *n*-hexane/Me_2CO (20:1) eluate, 180 mg]. Purification of fr. 3-1-1 by normal-phase pTLC using benzene/Me_2CO (4:1), followed by reversed-phase pTLC using Me_2CO/H_2O (3:1), to give kayeassamin E (**3**, 2 mg), a mixture of theraphins B and C (**15** and **16**, 2 mg), mammea B/AC cyclo F (**17**, 2 mg), and deacetylmammea E/BA cyclo D (**18**, 2 mg). Subfraction 3-1-2 was subjected to reversed-phase pTLC using MeCN/H_2O (7:3) to give a mixture (78 mg) and kayeassamin B (**9**, 26 mg). Purification of the mixture with semipreparative HPLC using MeCN/H_2O (7:3) solvent system [column: Discovery C-18, Supelco; flow rate: 5 mL/min] afforded kayeassamins F (**4**, 15.3 mg; t_R 38 min) and G (**5**, 3 mg; t_R 40 min). Subfraction 3-2 was subjected to a series of normal-phase pTLC using benzene/Me_2CO (5:1) and *n*-hexane/EtOAc (2:1) afforded kayeassamins A (**8**, 10.6 mg), H (**6**, 3.7 mg), and I (**7**, 25.2 mg). Subfraction 3-3 was purified by semipreparative HPLC with MeCN–H_2O (7:3) [column: Discovery C-18, Supelco; flow rate: 5 mL/min] to afford kayeassamins A (**8**, 90 mg; t_R 33 min), C (**1**, 9.6 mg; t_R 35 min), and D (**2**, 2 mg; t_R 38 min).

4.3.1. Kayeassamin C (1)

Pale yellow oil; $[\alpha]^{23}_{D}$ −60.87° (*c* 0.18, $CHCl_3$); UV, see Table 4; IR ($CHCl_3$) ν_{max} 3480, 1720, 1610, 1510, 1420, 1210, 1030, 930 cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 2; HREIMS *m/z* 456.2546 $[M]^+$ (calcd for $C_{27}H_{36}O_6$, 456.2512).

4.3.1.1. Preparation of (S)- and (R)-MTPA ester derivatives of 1. Two equal portions of **1** (each 2.5 mg) were dissolved in pyridine (250 μL) and (*R*)-MTPA-Cl (7.5 μL) or (*S*)-MTPA-Cl (7.5 μL) was added, respectively. The reaction mixtures were maintained at room temperature for 12 h. The reaction products were purified by normal-phase pTLC eluting *n*-hexane/EtOAc (3:1) afforded 1.8 mg each of **1a** and **1b**. ^1H NMR data of **1a** ($CDCl_3$, 400 MHz): δ_H 0.87 (H_3-3′), 1.72;1.95 (H_2-2′), 6.21 (H-3), 6.81 (H-1′); EIMS *m/z* 672 ($[M]^+$). ^1H NMR data of **1b** ($CDCl_3$, 400 MHz): δ_H 0.92 (H_3-3′), 1.77;1.99 (H_2-2′), 6.02 (H-3), 6.72 (H-1′); EIMS *m/z* 672 ($[M]^+$).

4.3.2. Kayeassamin D (2)

Pale yellow oil; $[\alpha]^{23}_{D}$ −47.90° (*c* 0.18, $CHCl_3$); UV, see Table 4; IR ($CHCl_3$) ν_{max} 1720, 1600, 1510, 1420, 1210, 1030, 930 cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 2; HREIMS *m/z* 456.2513 $[M]^+$ (calcd for $C_{27}H_{36}O_6$, 456.2512).

4.3.3. Kayeassamin E (3)

Pale yellow oil; $[\alpha]^{23}_{D}$ −9.79° (*c* 0.18, $CHCl_3$); UV, see Table 4; IR ($CHCl_3$) ν_{max} 1720, 1600, 1510, 1420, 1210, 1030, 930 cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 2; HREIMS *m/z* 374.1744 $[M]^+$ (calcd for $C_{21}H_{26}O_6$, 374.1729).

4.3.4. Kayeassamin F (4)

Pale yellow oil; $[\alpha]^{23}_{D}$ −9.49° (*c* 0.52, $CHCl_3$); UV, see Table 4; IR ($CHCl_3$) ν_{max} 1720, 1610, 1520, 1220, 1040, 930 cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 2; HREIMS *m/z* 388.1884 $[M]^+$ (calcd for $C_{22}H_{28}O_6$, 388.1886).

4.3.5. Kayeassamin G (5)

Pale yellow oil; $[\alpha]^{23}_{D}$ −33.23° (*c* 0.2, $CHCl_3$); UV, see Table 4; IR ($CHCl_3$) ν_{max} 1720, 1600, 1520, 1410, 1200, 1040, 925 cm^{-1}; ^1H and ^{13}C NMR, see Tables 1 and 2; HREIMS *m/z* 388.1924 $[M]^+$ (calcd for $C_{22}H_{28}O_6$, 388.1886).

4.3.6. Kayeassamin H (6)

Pale yellow oil; $[\alpha]_D^{23}$ −31.95° (*c* 0.06, $CHCl_3$); UV, see Table 4; IR ($CHCl_3$) ν_{max} 1730, 1600, 1520, 1430, 1380, 1220, 930 cm^{-1}; ^1H and ^{13}C NMR, see Table 3; HREIMS *m/z* 356.1669 $[M]^+$ (calcd for $C_{21}H_{24}O_5$, 356.1624).

4.3.7. Kayeassamin I (7)

Pale yellow oil; $[\alpha]^{23}_{D}$ −35.52° (*c* 0.90, $CHCl_3$); UV, see Table 4; IR ($CHCl_3$) ν_{max} 1720, 1610, 1410, 1210, 1020, 930 cm^{-1}; ^1H and ^{13}C NMR, see Table 3; HREIMS *m/z* 440.2191 $[M]^+$ (calcd for $C_{26}H_{32}O_6$, 440.2199).

4.3.8. Mammea B/AC (12)

^{13}C NMR ($CDCl_3$) δ_C 207.7 (C-1″), 163.9 (C-5), 160.3 (C-2), 160.1 (C-7), 159.7 (C-4), 156.6 (C-8a), 138.5 (C-3‴), 120.2 (C-2‴), 110.1 (C-3), 106.7 (C-6), 104.5 (C-8), 103.5 (C-4a), 46.6 (C-2″), 38.6 (C-1′), 25.9 (C-4‴), 22.8 (C-2′), 22.0 (C-1‴), 18.0 (C-5‴), 17.8 (C-3″), 13.9 (C-4″), 14.0 (C-3′).

4.4. In vitro preferential cytotoxicity

The in vitro preferential cytotoxicities of the crude extract and the isolated compounds were investigated using PANC-1 human pancreatic cancer cells under nutrient-deprived conditions according to the procedure described by Izuishi et al.[4] PANC-1 cells were seeded in 96-well plates (2×10^4 cells per well) and incubated in fresh Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceuticals; Tokyo, Japan) at 37 ℃ under 5% CO_2 and 95% air for 24 h. The nutrient-deprived medium (NDM) was prepared following the procedure described by Izuishi et al.[4] After the cells were washed with PBS (Nissui Pharmaceuticals), the medium was changed to either DMEM or NDM and serial dilutions of the test samples were added. For general preferential cytotoxicity assay, the cells were incubated for 24 h, then washed with PBS, and 100 μL of DMEM containing 10% WST-8 cell counting kit (Dojindo; Kumamoto, Japan) solution was added to the wells. After 2 h incubation, the absorbance at 450 nm was measured. The crude extracts were tested at 1, 10, 50, 100, and 200 μg/mL concentrations, while the pure isolates were tested ranging from 1 μM to 256 μM.

4.5. Time- and concentration-dependent in vitro preferential cytotoxicity

For time- and concentration-dependent experiments, the cells were incubated with the tested compounds for 3, 6, 9, 12, and 24 h. After incubation, morphological changes in PANC-1 cells were observed and photographs were taken under 20× magnification using phase-contrast microscopy (Olympus D-340L/C-840L Digital Camera, Tokyo, Japan). The cells were then washed with PBS, and 100 μL of DMEM containing 10% WST-8 cell counting kit (Dojindo; Kumamoto, Japan) solution was added to the wells. After 2 h incubation, the absorbance at 450 nm was measured. Cell viability was calculated from the mean values of data from three wells by using the following equation:

$$(\%)\ \text{Cell viability} = [\{\text{Abs}_{(\text{test sample})} - \text{Abs}_{(\text{blank})}\}/\{\text{Abs}_{(\text{control})} - \text{Abs}_{(\text{blank})}\}] \times 100$$

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