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Two New Compounds and Anti-complementary Constituents from *Amomum tsao-ko*

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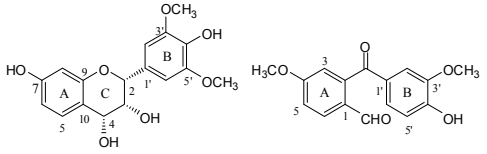
Two new compounds, (2*R*,3*R*,4*R*)-3',5'-dimethoxy-3,4,7,4'-tetrahydroxy-flavan (**1**) and 2-(4-hydroxy-3-methoxybenzoyl)-4-methoxy-benzaldehyde (**2**), together with 35 known phenolic compounds were obtained from the fruits of *Amomum tsao-ko*. Structures of the new compounds were elucidated on the basis of spectroscopic means, including 2D NMR, and high-resolution MS analysis. The isolated compounds were tested *in vitro* for their complement-inhibitory properties against the classical pathway (CP) and alternative pathway (AP). The results showed that 14 compounds exhibited anti-complementary activities against the CP and AP with CH_{50} values of 0.42 - 4.43 mM and AP_{50} values of 0.53 -1.51 mM. Preliminary mechanism studies showed that 1,7-bis(4-hydroxyphenyl)-4(*E*)-hepten-3-one (**8**) blocked C1q, C2, C3, C4, C5 and C9 components of the complement system, and hydroquinone (**15**) acted on C1q, C2, C3, C5 and C9 components.

Keywords: *Amomum tsao-ko*, Zingiberaceae, Complement inhibitor, Phenols, Diarylheptanoids, Benzenediols.

When activated inappropriately, the complement system may evoke pathologic reactions in a variety of inflammatory and degenerative diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), as well as acute respiratory distress syndrome (ARDS) [1, 2]. Therefore, inhibition of individual complement is a promising approach for the prevention and treatment of these diseases and numerous natural products have been reported to possess anti-complementary effect [3-5].

Amomum tsao-ko Crevost et Lemaire (Zingiberaceae) is an annual plant widely distributed in tropical regions of Asia. Its fruits are used under the name of "Cao-Guo" (Fructus Tsaoko) in traditional Chinese medicine (TCM) for the treatment of stomach disorders and infection of the throat, as well as a food flavor enhancer [6, 7]. In our effort to search for anti-complementary agents from TCMs, the ethanolic extract of *A. tsao-ko* fruits was found to show anti-complementary activity (CH_{50}: 0.75 ± 0.06 mg/mL, AP_{50}: 1.89 ± 0.35 mg/mL). Bioactivity-directed fractionation and isolation was thus performed with the ethanolic extract of *A. tsao-ko* fruits and led to the isolation of two new compounds, (2*R*,3*R*,4*R*)-3',5'-dimethoxy-3,4,7,4'-tetrahydroxy-flavan (**1**) and 2-(4-hydroxy-3-methoxybenzoyl)-4-methoxy-benzaldehyde (**2**) (Figure 1), together with 35 known ones (**3-37**). The isolates were tested for their *in vitro* anti-complementary activities against both the CP and AP, and the targets of the two most active compounds (**8** and **15**) on the complement activation cascade were also investigated.



1 **2**

Figure 1: Structures of **1** and **2**.

Compound **1** was obtained as a yellowish amorphous powder. Its molecular formula was determined as $C_{17}H_{18}O_7$ by HR-ESI-MS (*m/z* 357.0943 $[M + Na]^+$). Taken together with three oxygenated carbon signals at δ_C 86.6 (C-2), 72.2 (C-3) and 72.1(C-4) (Table 1), it is easily speculated that **1** possesses a flavan 3,4-diol skeleton, as is the case with melacacidin (2,3-*cis*-3,4-*cis*-flavan-3,3',4,4',7,8-hexaol) [8]. The key difference between **1** and melacacidin was that a symmetrical structure [δ_H 3.82 (3H, s, 3'-OCH_3), 3.81 (3H, s, 5'-OCH_3) and δ_H 6.68 (2H, s, H-2', 6')] was observed in ring B of **1**, while melacacidin possesses 3,4-disubstituted groups in ring B. The HMBC correlations between δ_H 6.68 (1H, s, H-2') in ring B and δ_C 86.6 (C-2) and δ_C 72.2 (C-3), and between δ_H 6.78 (1H, d, *J* = 8.3 Hz, H-5) and δ_C 72.2 (C-3) confirmed the flavanol skeleton of **1** (Figure 2A). The locations of methoxy groups at C-3'/5' and hydroxyl at C-4' were also verified by the HMBC correlations between δ_H 3.82 (3H, s, 3'-OCH_3) and δ_C 148.6 (C-3') and δ_C 149.2 (C-4'), and between δ_H 7.52 (1H, s, 4'-OH) and δ_C 149.2 (C-4'), respectively. The evidence for assignment of δ_H 7.14 (1H, s, 7-OH) was that the X-part [δ_H 6.98 (1H, d, *J*=2.0 Hz, H-8)] of the ABX system showed HMBC correlations with δ_C 110.5 (C-6), 147.5 (C-9) and δ_C 119.5 (C-10). The absolute configuration of **1** was determined as 2*R*,3*R*,4*R* by the negative *Cotton* effects at 240 nm and 274 nm in its CD spectrum [9], as well as the ROESY correlations of H- 2/H-3, and H-3/H-4 (Figure 2A). Therefore, the structure of compound **1** was determined as (2*R*,3*R*,4*R*)-3',5'-dimethoxy-3,4,7,4'-tetrahydroxy-flavan.

Compound **2** was obtained as colorless needles (PE-acetone, 5:1). The molecular formula was determined as $C_{16}H_{14}O_5$ by HR-ESI-MS (*m/z* 309.0736 $[M + Na]^+$). The ^{13}C NMR spectrum (Table 1) showed 12 carbon signals for two benzene rings (δ_C 153.1, 151.7, 148.2, 147.5, 128.9, 128.7, 126.2, 123.5, 115.4, 114.9, 111.1 and 110.7), one carbonyl at δ_C 196.3, one aldehyde at δ_C 191.2, and two overlapped methoxy groups (δ_C 55.6). The IR spectrum also showed the typical absorption band at 1612 cm^{-1} for benzene rings. The ^1H NMR spectrum of **2** (Table 1) showed two sets of ABX system proton signals at δ_H 6.85 (1H, d, *J* = 8.3 Hz), 7.42 (1H, d, *J* = 2.0 Hz) and 7.48 (1H, dd, *J* = 8.3, 2.0 Hz), as well as signals at δ_H 6.95 (1H, d, *J* = 8.3 Hz), 7.36 (1H, d, *J* = 2.0 Hz) and 7.40 (1H, dd,

Table 1: ^{1}H- and ^{13}C-NMR spectral data of compounds **1**[a] and **2**[b] (δ in ppm, *J* in Hz)

Position	1		2	
	δ_H (multiplicity)	δ_C	δ_H (multiplicity)	δ_C
1				148.2
2	4.66 (d, *J*=4.3)	86.6		128.7
3	4.20 (dd, *J*=4.3, 6.1)	72.2	7.36 (d, *J*=2.0)	110.7
4	3.79 (d, *J*=6.1)	72.1		153.1
5	6.78 (d, *J*=8.3)	134.1	7.40 (dd, *J*=8.3, 2.0)	126.2
6	6.82 (dd, *J*=8.3, 2.1)	110.5	6.95 (d, *J*=8.3)	115.4
7		152.6		
8	6.98 (d, *J*=2.1)	112.8		
9		147.5		
10		119.5		
1'		136.2		128.8
2'	6.68 (br. s)	104.3	7.42 (d, *J*=2.0)	111.1
3'		148.6		147.5
4'		149.2		151.7
5'		148.6	6.85 (d, *J*=8.3)	114.9
6'	6.68 (br. s)	104.3	7.48 (dd, *J*=8.3, 2.0)	123.5
1-CHO			9.75 (s)	191.2
4-OCH_3			3.82(s)	55.6
7-OH	7.14 (s)			
1'-COAr				196.3
3'-OCH_3	3.82 (s)	56.5	3.79 (s)	55.6
4'-OH	7.52 (s)		10.05 (br. s)	
5'-OCH_3	3.81 (s)	56.5		

[a] Measured in acetone-d_6 at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR.
[b] Measured in DMSO-d_6 at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR.

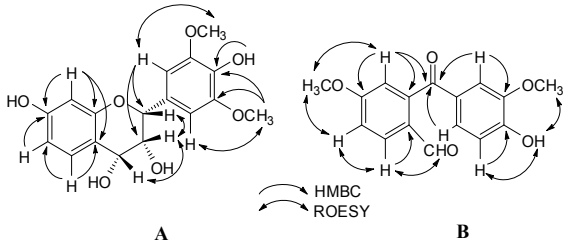


Figure 2: Key HMBC and ROESY correlations of **1** (A) and **2** (B).

2.0 Hz). The X-part of the ABX system in the A ring was deduced to be located at C-3 based on the HMBC correlations from δ_H 7.36 (1H, d, *J* = 2.0 Hz, H-3) to δ_C 148.2 (C-1), 153.1(C-4), 126.2 (C-5) and 196.3 (C-1') (Figure 2B). The methoxy group in the A ring was thus assigned at C-4 by the ROESY correlation between δ_H 7.36 (1H, d, *J* = 2.0 Hz, H-3) and the methyl signal at δ_H 3.79 (s, 3'-OCH_3). The aldehyde was assigned at C-1 by the ROESY correlation between the B-part of ABX system at δ_H 6.95 (1H, d, *J* = 8.3 Hz, H-6) and the aldehyde proton at δ_H 9.75 (1H, s, 1-CHO). Similarly, as shown in Figure 2B, the other ABX system and the locations of methoxy and hydroxyl in ring B were assigned. These two benzene rings were connected through one carbonyl group (δ_C 196.3, 1'-COAr), as indicated by the HMBC correlations between δ_H 7.36 (1H, d, *J* = 2.0 Hz, H-3), 7.42 (1H, d, *J* = 2.0 Hz, H-2'), 7.48 (1H, dd, *J* = 8.3, 2.0 Hz, H-6') and the carbonyl group (δ_C 196.3, 1'-COAr) (Figure 2B). Hence, the structure of compound **2** was established as 2-(4-hydroxy-3-methoxybenzoyl)-4-methoxy-benzaldehyde.

The 35 known phenolic compounds were identified by comparison of their ^{1}H, ^{13}C NMR and MS data with those reported in the literatures [10-20] as 4'-hydroxy-4-methoxychalcone (**3**), 4'-hydroxy-2'-methoxychalcone (**4**), 4,4'-dimethoxychalcone (**5**), 1,3-dimethoxybenzene (**6**), 4',7-dihydroxy-3',6-diprenylflavone (**7**), 1,7-bis(4-hydroxyphenyl)-4(*E*)-hepten-3-one (**8**), 4-hydroxy-2,5-dimethoxy-benzaldehyde (**9**), 3',7-dihydroxy-4'-methoxy-flavan (**10**), 2-methoxy-hydroquinone (**11**), 2',4'-dihydroxy-4- methoxy-chalcone (**12**), 5-indancarbaldehyde (**13**), 4-methoxybenzaldehyde (**14**), hydroquinone (**15**), 4-indancarbaldehyde (**16**), (-)-catechin

Table 2: Anti-complementary activities of the compounds from *A. tsao-ko* through the classical pathway (CH_{50}) and alternative pathway (AP_{50}) (mean ± SD, n=3)

Compounds	CH_{50} (mM)	AP_{50} (mM)
4'-Hydroxy-2'-methoxychalcone (**4**)	0.96 ± 0.15	NA[a]
1,7-Bis(4-hydroxyphenyl)-4(*E*)-hepten-3-one (**8**)	0.42 ± 0.15	0.66 ± 0.11
2-Methoxy-hydroquinone (**11**)	0.62 ± 0.09	0.86 ± 0.13
2',4'-Dihydroxy-4-methoxychalcone (**12**)	2.69 ± 0.42	NA
5-Indancarboxaldehyde (**13**)	1.33 ± 0.15	1.51 ± 0.38
Hydroquinone (**15**)	0.55 ± 0.11	0.53 ± 0.15
4-Hydroxy-2'-methoxychalcone (**23**)	4.43 ± 1.26	NA
Tsaokoarylone (**25**)	0.84 ± 0.07	0.72 ± 0.16
6,7-Dihydroxyindan-4-carbaldehyde (**28**)	0.66 ± 0.14	0.96 ± 0.13
3-Methoxy-catechol (**31**)	0.56 ± 0.13	0.54 ± 0.10
2-Methoxy-resorcinol (**32**)	0.64 ± 0.14	0.58 ± 0.14
4-(2-Hydroxypropyl)phenol (**34**)	0.93 ± 0.14	1.26 ± 0.18
Catechol (**36**)	0.58 ± 0.13	0.57 ± 0.15
4-Methoxy-catechol (**37**)	0.69 ± 0.16	0.64 ± 0.08
Heparin [b]	40 ± 14 [c]	97 ± 19 [c]

[a] NA: Not active. [b] Positive control. [c] μg/mL.

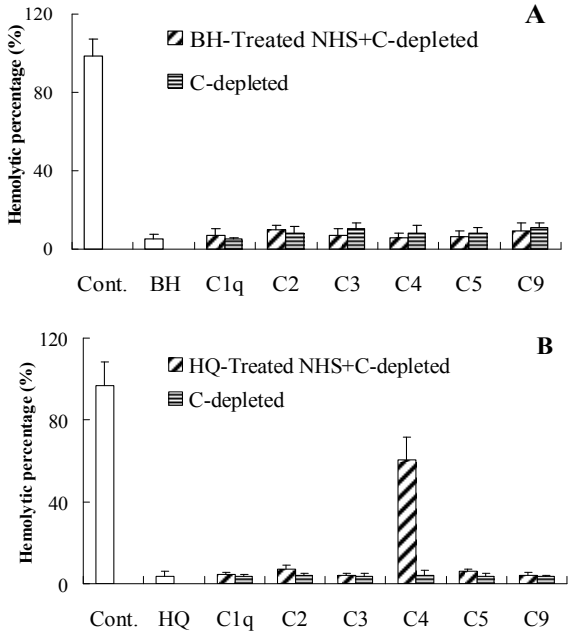


Figure 3: Targets of 1,7-bis(4-hydroxyphenyl)-4(*E*)-hepten-3-one (BH, A) and hydroquinone (HQ, B) on the complement activation cascade. BH-, HQ-treated sera were mixed with various complement-depleted (C-depleted) sera and the capacity of these C-depleted sera to restore hemolytic capacity in the CP was estimated by adding sheep antibody-sensitized erythrocytes. Cont., complement control group. Results are expressed as hemolytic percentages. Data are expressed as mean ± SD (n = 3).

(**17**), anisole (**18**), 2',4,4'-trimethoxychalcone (**19**), 4-(1-hydroxy-propyl)phenol-ethyl-4-hydroxy-(*S*)-benzenemethanol (**20**), abyssinoflavanone VII (**21**), 4-hydroxy-4'-methoxychalcone (**22**), 4-hydroxy-2'-methoxychalcone (**23**), 2-methoxy-benzaldehyde (**24**), tsaokoarylone (**25**), 3-methoxy-benzaldehyde (**26**), 3-hydroxy-4-methoxybenzaldehyde (**27**), 6,7-dihydroxy-4-indancarbaldehyde (**28**), 3-methoxy-4-hydroxy-benzaldehyde (**29**), 6-hydroxy-4-aldehydeindene (**30**), 3-methoxy-catechol (**31**), 2-methoxy-resorcinol (**32**), 3,5-dihydroxybenzoic acid (**33**), 4-(2-hydroxy-propyl)phenol (**34**), 3-hydroxybenzoic acid (**35**), catechol (**36**), and 4-methoxy-catechol (**37**),. Except for compounds **8**, **14**, **15**, **17**, **18**, **25**, **29** and **36**, all the other compounds were obtained from this species for the first time.

The isolated compounds (purity>90%, by HPLC analysis), except **33** and **35**, were evaluated for their anti-complementary activities against both CP and AP. As shown in Table 2, fourteen compounds possessed anti-complementary effects against the CP with CH_{50} values of 0.42 - 4.43 mM. On the AP, eleven isolates were found

active, with AP_{50} values of 0.53 - 1.51 mM. Compounds **8**, **15**, **31**, **32**, **36** and **37** showed good activities against both CP and AP with CH_{50} and AP_{50} values less than 0.7 mM. Derivatives of chalcone (**4**, **12** and **23**) were inactive against AP while they demonstrated inhibitory activity against CP. As the two most active compounds against the CP and AP respectively, 1,7-bis(4-hydroxyphenyl)-4(*E*)-hepten-3-one (**8**) and hydroquinone (**15**) were selected for the preliminary mechanism study. When treated with 1,7-bis(4-hydroxyphenyl)-4(*E*)-hepten-3- one (**8**), hemolytic percentages of the C1q-, C2-, C3-, C4-, C5- and C9-depleted sera were 5.04 ± 1.03%, 8.33 ± 2.98%, 9.46 ± 3.00%, 8.36 ± 3.78%, 8.21 ± 2.43% and 9.10 ± 2.19%, respectively (Figure 3A), indicating that **8** interacted with C1q, C2, C3, C4, C5 and C9. Meanwhile, hydroquinone (**15**) acted on C1q, C2, C3, C5 and C9 components (Figure 3B).

Experimental

Reagents: Sheep erythrocytes were collected in Alsevers' solution. Normal human serum (NHS) was obtained from healthy male donors (average age of 20 years). Rabbit erythrocytes were obtained from the ear vein of New Zealand white rabbits. Heparin (sodium salt, 160 IU/mg) was purchased from Shanghai Aizite Biotech Co. Ltd. (Shanghai, China). Veronal buffer saline (VBS, pH 7.4) contained 0.5 mM Mg^{2+} and 0.15 mM Ca^{2+}(VBS^{2+}), and VBS 5 mM Mg^{2+} and 8 mM EGTA (VBS-Mg-EGTA). Anti-C1q, Human (Goat); Anti-C2, Human (Goat); Anti-C5, Human (Rabbit) and Anti-C9, Human (Goat) were purchased from Merck Biosciences (Darmstadt, German). Anti-C3, Human (Goat) and Anti-C4, Human (Goat) were purchased from Shanghai Sun Biotech Co. Ltd. (Shanghai, China).

Plant material: The fruits of *A. tsao-ko* were purchased from Huayu Materia Medica Co., Ltd. (Shanghai, China) in October 2007. The plant material was verified by Dr Daofeng Chen. A voucher specimen (DFC-CG-H2003050607) has been deposited in the Herbarium of Materia Medica, Department of Pharmacognosy, School of Pharmacy, Fudan University, Shanghai, China.

Extraction and isolation: The air-dried fruits (15.0 kg) were extracted with 95% ethanol (10 L × 5 times) at room temperature. The extracts were combined and concentrated under reduced pressure, and the resulting residue (319.2 g) was resuspended in 2.0 L of water and successively partitioned with PE (60-90ºC), ethyl acetate, and *n*-butanol (each 2 L × 4 times). The *n*-butanol fraction was found to show the highest activity. Therefore, the active *n*-butanol fraction was subjected to AB-8 macroporous resin chromatography eluted with 30%, 50%, 70% and 90% aqueous ethanol to afford 4 subfractions. The subfractions obtained with 50% and 70% ethanol were found to show obvious anti-complementary activities and were thus further chromatographed over a silica gel column (CC).

The 50% EtOH subfraction (21.3 g) was subjected to CC (silica gel, PE-acetone, from 100:0 to 1:1) to yield F-1 - F-8. F-2 (1.7 g) was separated by CC (silica gel, PE-ethyl acetate, from 20:1 to 10:1) and then purified over Sephadex LH-20 (chloroform-methanol, 1:1) to afford **13** (21.2 mg). F-3 (1.2 g) was purified over CC (silica gel, PE-ethyl acetate, 10:1) to give **16** (12.7 mg), **22** (8.0 mg), **26** (4.3 mg), **34** (10.2 mg), **9** (14.3 mg) and **10** (5.8 mg). F-5 (2.5 g) was loaded onto a column of silica gel and eluted with PE-ethyl acetate, from 10:1 to 5:1, and then subjected to Sephadex LH-20 (chloroform-methanol, 1:1) and preparative TLC (chloroform-methanol, 20:1) to afford **2** (15.4 mg), **3** (4.4 mg), **4** (3.9 mg), **14** (4.1 mg), **21** (12.1 mg) and **23** (16.6 mg). F-6 (1.1 g) was purified by CC (silica gel, PE-acetone, from 5:0 to 5:1) and then subjected to gel filtration over Sephadex LH-20 (chloroform-methanol, 1:1) and preparative TLC (chloroform-methanol, 15:1) to afford **31** (8.7 mg), **32** (12.1 mg), **36** (9.5 mg), **27** (3.3 mg), **30** (4.4 mg) and **35** (3.9 mg). F-7 (1.8 g) was purified by CC (silica gel, PE-acetone, from 10:1 to 5:1) and then subjected to preparative TLC (chloroform-methanol, 10:1) to furnish **11** (15.3 mg), **15** (6.8 mg), **33** (9.3 mg) and **37** (13.8 mg).

The 70% EtOH subfraction (13.7 g) was subjected to CC (silica gel, PE-acetone, from 100:0 to 1:1) to yield F-9 - F-16. F-11 was further separated by CC (silica gel, chloroform-methanol, 10:1) and then purified over Sephadex LH-20 (chloroform-methanol, 1:1) and preparative TLC (chloroform-methanol, 15:1) to give **20** (9.9 mg), **24** (9.3 mg) and **25** (17.9 mg). F-12 (1.5 g) was chromatographed over Sephadex LH-20 (methanol) and then separated by CC (silica gel, chloroform-methanol, 15:1) to yield **1** (5.3 mg), **7** (5.1 mg), **8** (7.9 mg) and **18** (6.3 mg). F-13 (1.6 g) was chromatographed over a column of silica gel, eluting with chloroform-methanol, 15:1), and then purified over Sephadex LH-20 (methanol) to afford **12** (7.2 mg), **17** (7.7 mg), **28** (5.3 mg) and **29** (10.9 mg). F-14 (2.7 g) was purified by CC (silica gel, chloroform-methanol, 7:1) and then purified over Sephadex LH-20 (chloroform-methanol, 1:1) to afford **5** (8.3 mg), **6** (5.9 mg) and **19** (6.3 mg).

(2*R*,3*R*,4*R*)-3',5'-Dimethoxy-3,4,7,4'-tetrahydroxy-flavan (**1**)
Pale yellow powder.
MP: 182-184ºC.
$[\alpha]_D^{25}$: - 26.8 (*c* 0.02, acetone).
IR (KBr) ν_{max}: 3420, 2955, 2920, 2850, 1634, 1558, 1446, 1384, 1303 cm^{-1}.
UV (acetone): λ_{max} (log ε): 223 (5.2), 255 (3.1, sh) nm.
CD (*c* 0.1, methanol): nm($\Delta\varepsilon$): 240 (-32.1), 274 (-11.4).
^1H NMR and ^{13}C NMR (acetone-d_6): Table 1;
HR-ESI-MS: *m/z* 357.0943 ([*M* + Na]$^+$, $C_{17}H_{18}O_7Na^+$, calc. 357.0945).

2-(4-Hydroxy-3-methoxybenzoyl)-4-methoxy-benzaldehyde (**2**)
Colorless needles (PE-acetone, 5:1).
MP: 166-167ºC.
IR (KBr) ν_{max}: 3416, 1721, 1612, 1462, 1383, 1272 cm^{-1}.
UV (acetone): λ_{max} (log ε): 223 (4.6), 250 (2.7, sh) nm.
^1H NMR and ^{13}C NMR (DMSO-d_6): Table 1.
HR-ESI-MS: *m/z* 309.0736 ([*M* + Na]$^+$, $C_{16}H_{14}O_5Na^+$, calc. 309.0734).

Anti-complementary activity through the classical pathway: The anti-complementary activities through the classical pathway (CP) were measured as described previously [21]. Briefly, sensitized erythrocytes (EAs) were prepared by incubation of sheep erythrocytes (4.0×10^8 cells/mL) with equal volumes of rabbit anti-sheep erythrocyte antibody in VBS^{2+}. Samples and heparin (positive control) were individually dissolved in VBS^{2+}. Normal human serum (NHS) was used as the complement source. The 1:80 diluted serum of Guinea pig was chosen to give sub-maximal lysis in the absence of complement inhibitors. Serial dilutions of the test samples (100 µL) were preincubated with a mixture of 100 µL NHS and 200 µL VBS^{2+} at 37°C for 10 min, followed by adding 200 µL EA to the mixture and co-incubating at 37°C for 30 min. The different assay controls were incubated under the same conditions: (1) vehicle control, 200 µL EAs in 400 µL VBS^{2+}; (2) control 100% lysis, 200 µL EAs in 400 µL water; (3) sample control, 100 µL dilution of each sample in 500 µL VBS^{2+}. After reaction, the resulting mixture was centrifuged immediately, and the optical density of the supernatant was then measured at 405 nm with a spectrophotometer (Wellscan MK3, Labsystems Dragon). The

absorbance of sample (As), sample control (Asc) and 100% lysis control (Al) were obtained.

Anti-complementary activity through the alternative pathway: The anti-complementary activities through the classical pathway (CP) and alternative pathway (AP) were measured according to Klerx's method [22]. Briefly, each sample was dissolved in EGTA-VB, and serial dilutions of the samples were prepared by adding appropriate volumes of EGTA-VB. After pre-incubation of each sample (150 μL) with 1:10 diluted NHS (150 μL) at 37°C for 10 min, 200 μL rabbit erythrocytes (ERs 3.0×10^8 cells/mL) were added, followed by a second incubation at 37°C for 30 min. After reaction, the resulting mixture was centrifuged immediately, and the optical density of the supernatant was then measured at 405 nm.

Identification of the targets on the complement activation cascade: Tests to identify the targets of the complement activation cascade were conducted according to Xu's method [5]. Various dilutions of each antiserum were incubated with the same volume of NHS (1:10, v/v) at 37°C for 15 min. After centrifugation, the supernatant (200 μL) was incubated with 200 μL VBS^{2+} and 200 μL EAs, and cell lysis was measured. The antiserum dilution against the NHS hemolytic capacity was then determined. The optimal dilutions (1:1 for C3 and C4; 1:32 for C5, and 1:64 for C1q, C2, and C9, v/v) were incubated with the same volume of NHS (1:10, v/v) at 37°C for 15 min, followed by centrifugation, and the supernatants were collected and stored as complement-depleted (C-depleted) sera in aliquots at -70°C before use in hemolytic assays.

Capacity of depleted sera to lyse EAs through the CP was assessed in the presence or absence of sample-treated NHS. Sample-treated NHS was obtained by incubating an optimally diluted sample with an equal volume of 1:10 (v/v) diluted NHS at 37°C for 10 min. The examined concentrations of 1,7-bis(4-hydroxyphenyl)-4(*E*)-hepten-3-one (**8**) and hydroquinone (**15**), just sufficient to cause complete loss of hemolytic activity of 1:10 diluted NHS, were 1.12 mM and 1.24 mM. For the target complement group (the assay of capacity of various depleted sera to restore the hemolytic capacity of sample-treated serum), 200 μL EAs and 200 μL individual depleted sera of C1q, C2, C3, C4, C5 or C9 were added to 200 μL sample-treated NHS and the mixture was incubated at 37°C for 30 min. After centrifugation and measurement of the optical density of the supernatant, the percentage of hemolysis was calculated. For the assay of the individual depleted serum group, C-depleted sera were directly incubated with EAs under the same conditions, and the hemolytic activities were calculated. The controls: (1) vehicle control: 200 μL EAs in 400 μL VBS^{2+}; (2) 100% lysis: 200 μL EAs in 400 μL water; (3) complement control: 100 μL NHS (1:10, v/v) and 200 μL EAs in 300 μL VBS^{2+}; and (4) sample control, 100 μL sample in 500 μL VBS^{2+}, were incubated under the same conditions.

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