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In Vitro Anticomplementary Activity of Phenylpropanoids from *Agastache rugosa*

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Abstract—In searching for anticomplementary compounds, three phenylpropanoids were isolated from the roots of *Agastache rugosa* and identified as rosmarinic acid (RA), rosmarinic acid methyl ester (RAM) and caffeic acid methyl ester (CAM) by NMR analyses. RA and RAM exhibited strong inhibitory activity on both the classical pathway (CP) and the alternative pathway (AP) of the complement system, *in vitro*, but CAM did far less than RA and RAM. RAM-M1~-M5, the methylated derivatives from the RAM, showed that the inhibitory activity was decreased in inverse proportion to the number of methylated groups and RAM-M2~-M4, the isomers of two methylated hydroxyl groups, exhibited different inhibition activity.

Key words—*Agastache rugosa*; rosmarinic acid; rosmarinic acid methyl ester; caffeic acid methyl ester; anticomplement activity.

The complement system is a major effector of humoral immunity involved in the host defence system. It is activated through antigen-antibody mediated process (classical pathway, CP) and/or antibody independent process (alternative pathway, AP).[1] The activated components, normally, mediate protective process of inflammation against foreign invasive organisms and other antigens.[2] However, active fragments (C3a, C4a and C5a) of the components associate with a variety of diseases[3~6] and act fatally on organ transplantation.[7] Therefore specific inhibitors to modulate complement activation should be useful in therapy of diseases caused by inappropriate complement activation.

We have been screening anticomplementary compounds from medicinal plants and isolated three active compounds from the roots of *Agastache rugosa*, which has been used not only for spices on various foods but also for traditional medicine against intestinal disorder, emesis, and diarrhea in Korea.

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EXPERIMENTAL

Buffers—The components of gelatin veronal buffer(GVB) were as follows : 1.8 mM sodium barbital, 3.1 mM barbituric acid, 0.1% gelatin, 0.141 M NaCl, and 0.3% sodium azide (pH 7.3). 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$ were added to GVB for the CP (GVB^{2+}) and 8 mM $MgCl_2$, and 4 mM ethylene glycol-bis(β-aminoethyl ether)-N,N,N,'N'-tetraacetic acid (EGTA) were added to GVB for the AP (Mg-EGTA-GVB).

Complement titration—The overall procedure was based on the methods by Kabat

et al.[8] for the CP and Platts-Mills *et al*[9] for the AP of the complement system. For the CP assay, sheep erythrocyte suspension (5.0×10^6 cells/ml) was sensitized by addition the equal volume of 1/50~1/200 diluted hemolysin (S-1389, Sigma Co), and incubation at 37°C for 30 min. Afterwards, sensitized erythrocytes (EA) were kept at 4°C and restored to former concentration prior to use. For the AP assay, rabbit erythrocyte suspension (5.0×10^8 cells/ml) was used. Human complement serum from healthy volunteer was diluted to 1/50~1/100 for the CP assay and 1/5~1/20 for the AP assay, respectively, in the corresponding buffer. Test samples were dissolved in DMSO and diluted to 1/40 with each buffer. All the prepared reagents were stored at 4°C before titration.

The titration procedure was based on the methods by Klerx *et al*,[10] and as follows : After 80 μl of complement solution was mixed with 80 μl of corresponding buffer with or without a sample on the microtiter plate, the mixture was preincubated at 37°C for 30 min and then added 40 μl of sensitized erythrocytes (EA) for the CP assay or 40 μl rabbit erythrocyte suspension for the AP assay, respectively. After incubated with shaking at 37°C for 30 min, the reaction mixture was centrifuged (800×g, 4°C, 4 min) immediately and 100 μl of the supernatant was transferred to another microplate for measuring optical density at 540 nm using microplate reader.

The controls for maximum and background lysis were measured by reaction with H_2O and preheated serum (56°C, 30 min), respectively, instead of normal complement. The dilution factors of the hemolysin (CP) and complement (CP and AP) were determined to obtain 50% hemolysis in our experimental condition.

The % hemolysis was calculated by $(A_i / A_{max})\times100\%$ where Ai and Amax indicated the mean of triplicate OD_{540nm} values of a given titration and maximum lysis by H_2O from the corresponding background, respectively. The anticomplementary activity was expressed by $[1-(\%\ \text{hemolysis}_{sample} / \%\ \text{hemolysis}_{standard})]\times100\%$.

Plant materials – The roots of *Agastache rugosa* were collected in Yangsan, Korea. After dried in the dark side, they were chopped finely for extraction. The voucher specimen is deposited in our laboratory.

Instruments – The optical density for detecting hemolysis was measured by using Emax® precision micorplate reader (Molecular Devices Co.) at 540 nm. UV spectra were obtained from Milton Roy Spectronic 3000 array and NMR spectra (^1H, ^{13}C, C-H COSY, DEPT and COLOC) were measured by Varian UNITY 300.

Extraction and isolation – Dried roots (4.5 kg) were extracted with 90% methanol (10 *l* ×3) at room temperature and the extract was concentrated to 1.5 *l* under reduced pressure. The concentrated aqueous residues were partitioned successively with n-hexane, ethyl acetate and n-butanol (500 ml×4, each), respectively.

Activity-guided separation of the ethyl acetate fraction, which showed strong anticomplementary activity in preliminary screening, was carried out with a series of column chromatography (silica gel : chloroform-methanol mixture gradient: 15:1 → 1:1 v/v, RP-18: methanol-water mixture: 7:3 v/v and Sephadex LH-20 : chloroform-methanol mixture 2:1 → 100% methanol). Three active compounds, AR1, AR2 and AR3 were isolated and purified using HPLC [RP-18, φ10×250 mm, AR1 (33 mg): methanol-

water 7:3, AR2 (850 mg): methanol-water 65:35 and AR3 (415 mg): acetonitrile-phosphate buffer (0.1 M, pH 3.5), 35:65, v/v] from the each subfraction, respectively.

O-methylation of rosmarinic acid methyl ester (RAM) - Compound AR2 (390 mg) was treated with diazomethane to give methylated derivatives and RAM-M1, -M2, -M3, -M4 and -M5 were respectively isolated by prep. TLC (silica gel GF_{254}, R_f values: RAM-M1 0.50, -M2 0.58, -M3 0.70, -M4 0.85, -M5 0.90 in chloroform-methanol 10:1, v/v) and purified by HPLC (RP-18, φ10×250 mm, methanol-water 7:3, v/v).

RESULTS AND DISCUSSION

Structure analysis According to activity-guided separation, AR1, AR2 and AR3 were isolated and identified as caffeic acid methyl ester (CAM), rosmarinic acid methyl ester (RAM) and rosmarinic acid (RA), respectively, by NMR data analyses (Table I, Fig 1). The structures of RAM-M1~-M5 were analyzed by ^{1}H-NMR and ^{13}C-NMR. RAM-M1 showed the presence of one methyl ether group (δ_H 3.87, s; δ_C 56.40). RAM-M2, -M3 and -M4 were the position isomers of two methyl ether groups (RAM-

Table I. NMR data of compounds AR1, AR2 and AR3 from *Agastache rugosa*[a]

carbon No.	AR1			AR2			AR3		
	carbon	proton	COLOC	carbon	proton	COLOC	carbon	proton	COLOC
1	127.70(C)[c]	-	-	127.41(C)	-	-	127.66(C)	-	-
2	115.12(CH)	7.03(s)[d]	C4, C6, C7	115.15(CH)	7.06(d, 1.8)	C4, C6, C7	115.23(CH)	7.05(d, 2.1)	C4, C6, C7
3	146.12(CH)	-	-	146.56(C)	-	-	146.79(C)	-	-
4	146.83(C)	-	-	149.55(C)	-	-	149.72(C)	-	-
5	116.51(CH)	6.77(d, 8.1)	C1, C3	116.38(CH)	6.80(d, 8.1)	C1, C3	116.51(CH)	6.78(d, 8.1)	C1, C3
6	122.94(CH)	6.94(dd, 15×8.1)	-	123.13(CH)	6.95(dd, 1.8×8.1)	C7	123.20(CH)	6.95(dd, 2.1×8.1)	C7
7	146.95(CH)	7.54(d, 15.9)	C9	147.84(CH)	7.57(d, 15.9)	C8, C9	147.75(CH)	7.56(d, 15.9)	C8, C9
8	114.83(CH)	6.25(d, 15.9)	C1	114.01(CH)	6.27(d, 15.9)	C1, C9	114.41(CH)	6.27(d, 15.9)	C1
9	169.77(C)	-	-	168.23(C)	-	-	168.48(C)	-	-
1′				128.60(C)	-	-	129.27(C)	-	-
2′				117.41(CH)	6.74(br. s)	C3′, C6′	117.60(CH)	6.76(d, 1.8)	C3′
3′				145.14(C)	-	-	145.27(C)	-	-
3′				145.96(C)	-	-	146.15(C)	6.71(d, 8.1)	-
4′				116.20(CH)	6.72(d, 8.1)	C4′	116.32(CH)	6.62(dd, 1.8×8.1)	C4′
5′				121.72(CH)	6.58(dd, 1.5×8.1)	C4′	128.84(CH)	3.11(dd, 4.5×14.1)	-
6′				37.74(CH_2)	3.04(m)	C8′, C1′	37.90(CH_2)	3.01(dd, 8.1×14.1)	C9′
7′								5.20(dd, 4.5×8.1)	
				74.51(CH)	5.21(dd, 7.2×5.7)	C9′, C1′	74.61(CH)	-	C9′
8′				172.05(C)	-	-	173.53(C)		-
9′									
9-OCH_3	52.02(CH_3)	3.75(s)	C9	52.64(CH_3)	3.70(s)	C9′			
9′-OCH_3									

[a] Measured in CD_3OD at 300 MHz(^{1}H) and 75 MHz(^{13}C, DEPT, C-H COSY and COLOC) with TMS as an internal standard.
[b] Correlation spectroscopy for long range coupling.
[c] Each carbon charactor was determined by DEPT.
[d] Coupling patten and constant in Hz.

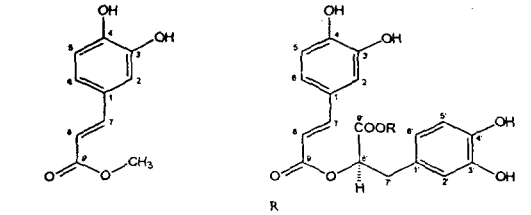

Fig. 1. Structures of compounds isolated from *Agastache rugosa*.

M2: δ_H 3.81, 3.82 each s; δ_C 56.39, 56.47, -M 3: δ_H 3.81, 3.86 each s; δ_C 56.39, 56.42 and - M4: δ_H 3.86×2 each s; δ_C 56.39, 56.45, respectively). RAM-M5 was appeared to have three methyl ether groups (δ_H 3.79, 3.81 and 3.86 each s; δ_C 56.45, 56.60 and 56.63).

RA has been reported to occur in several species of the family Labiatae[11,12] but it was first example from *Agastache* species. RAM and CAM, as similar to ethyl lithospermate,[13] might be methylated during the extraction and isolation procedure from rosmarinic acid and caffeic acid, respectively. They were confirmed by HPLC with authentic compounds after mild alkali treatment.

Anticomplementary activity RA exhibited the highest inhibitory activities on the CP and the AP of the complement system. On the CP, RA showed that the inhibition of hemolysis was 5.4± 3.6% at 0.063 mM and it was rapidly increased in the range of 0.063 mM~0.5 mM, then came up to 95.8± 0.2% at 0.5 mM. RA inhibited the AP more effectively than the CP below 0.063 mM but the inhibition was reversed above 0.25 mM (Fig. 2, 3). RA has been reported to inhibit leukotriene B_4 (LTB_4) and 5-hydroxy-6, 8, 11, 14-eicosatetraenoic acid (5-HETE) on arachidonate metabolism,[14] lipid peroxidation,[15] C3 convertase and the formation of C5a in the complement system.[16]

RAM, of which carboxyl group was masked by methyl group, showed strong inhibitory activities next to RA. The inhibitory activity of RAM was dose-dependent and higher on the CP than on the AP at any concentration. CAM exhibited very weak inhibitory activity on the CP below 0.5 mM, but the activity was markedly

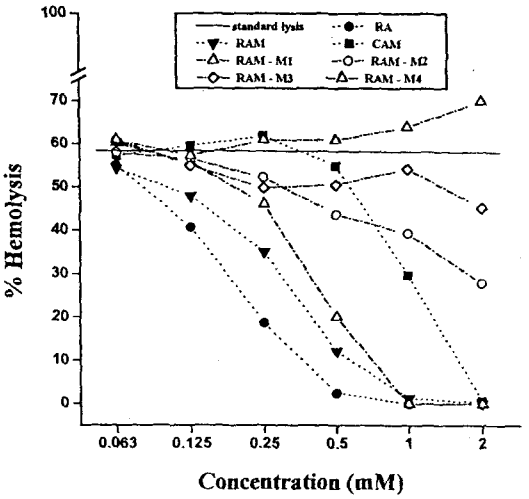

Fig. 2. Anticomplementary effect of isolated compounds from *Agastache rugosa* and four derivatives of RAM on the CP of complement system.

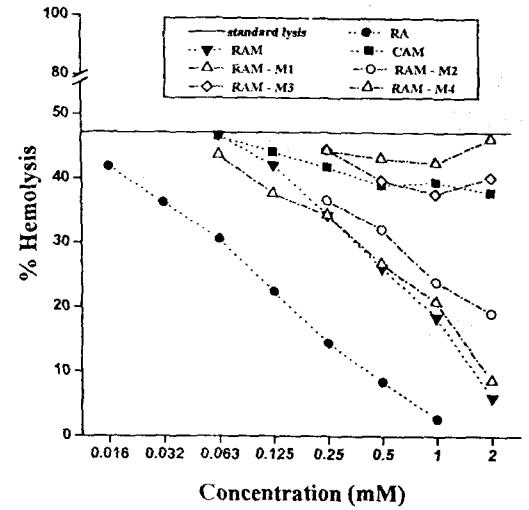

Fig. 3. Anticomplementary effect of isolated compounds from *Agastache rugosa* and four derivatives of RAM on the AP of the complement system.

Table II. Anticomplementary activity of isolated compounds from *Agastache rugosa* and methylated derivatives of rosmarinic acid methyl ester (RAM).

Compound[a]	Inhibition (%)	
	Classical pathway (CP)	Alternative pathway (AP)
Rosmarinic acid	95.8±0.2[b]	79.6±1.7
Rosmarinic acid methyl ester	79.1±3.1	57.3±5.7
Caffeic acid methyl ester	5.0±2.5	17.8±1.6
RAM-M1	65.2±4.8	41.8±7.2
RAM-M2	24.6±4.2	31.3±1.3
RAM-M3	12.9±2.9	15.0±5.1
RAM-M4	-5.2±3.3[c]	7.8±1.3
RAM-M5	-3.3±5.5[c]	4.7±6.8

[a] Concentration 0.5mM
[b] Means±S.D. of triplicate experiments
[c] Implied activation

increased from 0.5 mM to 2.0 mM and was 98.8±0.5% at 2.0 mM. The inhibitory activity of CAM on the AP was very low, as 35.7±6.4% at 2.0 mM. RAM-M1~-M5, the methylated compounds of hydroxyl groups from RAM, exhibited that the inhibitory activities decreased in inverse proportion to the number of methoxyl groups and RAM-M2~-M4, which had two methoxyl groups, exhibited different activities. RAM-M1 showed that the inhibition pattern was similar to RAM on the CP and somewhat low on the AP, respectively. RAM-M2 showed that the inhibition was dose-dependent but far less than that of RAM-M1. RAM-M3 showed very weak inhibition on both pathways. RAM-M4 and -M5 showed promotion of hemolysis by dose-dependent on the CP and little activity on the AP (Fig 2, 3 and Table II).

These results were in accordance with the report of K. Cimanga *et al.*[17] that the inhibitory activity was increased with the number of hydroxyl groups and the position of hydroxyl groups was important in quercetin and kaempferol. Thus it was considered that the phenolic hydroxyl groups of rosmarinic acid were more important than carboxyl group to inhibit the complement system and position of free hydroxyl groups could be related to the inhibitory activity of the complement system.

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