Antioxidant Constituents of *Caragana tibetica*

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Caragana tibetica Kom. (Fabaceae) is a medicinal plant that has been traditionally used in western part of China. In the course of our screening study on antioxidant activity of medicinal plants, the 70% acetone extract of the stems of *C. tibetica* was found to have a potent superoxide anion scavenging activity. Tibeticanol (1), a new piceatannol dimer possessing antioxidant activity, was isolated along with eleven known aromatic compounds. Their structures were elucidated on the basis of NMR and MS data. Enzyme oxidation of monomeric stilbene, piceatannol (3), with horseradish peroxidase and hydrogen peroxide yielded cassigarol E (5) and G (6) as major products. Most of the isolated compounds exhibited superoxide anion scavenging activity.

Key words *Caragana tibetica*; stilbenoid; tibeticanol; constant time-HMBC; horseradish peroxidase; superoxide anion scavenging activity

Fabaceae plant *Caragana tibetica* Kom. has been traditionally used in Tibet, Sichuan, Yunnan and Gansu area of China to treat atherosclerosis, hyperlipidemia, hypertension and arthritis.[1] During the course of study on antioxidant compounds contained in Chinese medicinal plants, 70% acetone extract of the stems of *C. tibetica* was found to exhibit a potent superoxide anion scavenging activity. The extensive fractionation of the 70% acetone extract afforded a new piceatannol dimer, tibeticanol (1), along with eleven known compounds, resveratrol (2),[2] piceatannol (3),[3,4] isorhapontigenin (4),[4] cassigarol E (5),[5] cassigarol G (6),[5] kompasinol A (7),[6,7] scirpusin A (8),[8] scirpusin B (9),[8] sativan (10),[9] (+)-(6as, 11as)-medicarpin (11)[10] and syringaresinol (12).[11] Their structures were identified on the basis of NMR and MS data. The NMR spectra of the known compounds, 2—12 were identical to those reported in the literatures. The reported structures of cassigarol E and cassigarol G were deduced by biogenetic consideration and lacked rigid evidences on the proposed benzodioxane structures.[5] In this study, the C–O bonds between two piceatannol moieties in cassigarol E (5) and cassigarol G (6) have been unambiguously established by Constant Time-HMBC (CT-HMBC) analysis.[12] Phenol oxidative coupling reaction of piceatannol (3) with horse radish peroxidase (HRP) and hydrogen peroxide gave cassigarol E (5) and cassigarol G (6) as main reaction products.

Results and Discussion

Compound 1 was obtained as amorphous brown powder. High Resolution FAB-MS (HR FAB-MS) gave a $[M-H]^-$ peak at *m/z* 485.1261 corresponding to the molecular formula $C_{28}H_{22}O_8$ indicating a dimer of a simple stilbene, piceatannol (3). The ^{13}C-NMR gave 28 aromatic carbon signals. The ^{1}H-NMR spectrum showed two pairs of *trans* olefinic protons at δ 6.74 (1H, d, *J*=16.1 Hz, H-β), 6.34 (1H, d, *J*=16.1 Hz, H-α), 6.59 (1H, d, *J*=16.3 Hz, H-β'), 6.49 (1H, d, *J*=16.3 Hz, H-α'); protons of an AMX aromatic system at δ 6.61 (1H, d, *J*=8.2 Hz, H-5'), 6.60 (overlapped, H-2'), 6.51 (1H, dd, *J*=8.2, 1.7 Hz, H-6'); protons of an AX_2 system at δ 6.00 (1H, br s, H-4''), 6.08 (2H, d, *J*=1.7 Hz, H-2''' and H-6'''), protons of an AX system at δ 6.63 (1H, d,

J=1.7 Hz, H-6), 6.30 (1H, d, *J*=1.7 Hz, H-4), and two singlet signals at δ 7.17 (1H, s, H-3'') and 6.41 (1H, s, H-6''). The ^{1}H-NMR data suggest that 1 is a dimer of piceatannol (3), in which two monomeric moieties form a C–C bond between the aromatic rings of piceatannol (3). The correlation observed between H-6''' (6.41, s) and C-2 (118.02) in the HMBC spectrum (shown with a thick arrow in Fig. 1) indicates that the C-2 of one piceatannol moiety is linked to C-1'' of the other piceatannol moiety. Together with the other correlations observed in the HMQC and HMBC spectra, the structure was unambiguously established to be 1. Since it is a new compound, 1 is called tibeticanol. ^{1}H- and ^{13}C-NMR spectral data are summarized in Table 1.

The acetylated cassigarol E (5) and cassigarol G (6) were isolated from the acetylated fraction of the extracts of *Cassia garrettiana*,[5] however the mode of linkages between the two piceatannol moieties were deduced by biogenetic consideration. The HMBC correlations between two monomeric moieties are essential to determine their structures unambiguously, however normal HMBC measurements failed to detect the definitive correlations. Constant Time-HMBC method was then applied to the structure determination of cassigarol E (5) and cassigarol G (6). The correlations between H-α and C-3'', H-β and C-4'' rigorously established that the α and β carbons of piceatannol ethylene group are bonded to the catechol hydroxyl groups of the other moiety. These decisive correlations in CT-HMBC are shown in thick arrows in Fig. 2.

Stilbene oligomers were reported to have a wide variety of structures.[13] These oligomers are regarded to be formed by oxidative coupling reaction of phenolic groups of monomeric
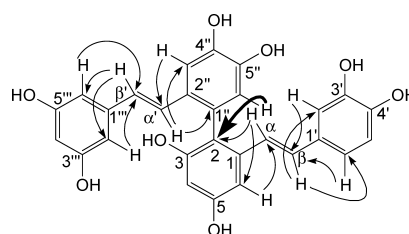

Fig. 1. Significant HMBC Correlations Observed in 1

Table 1. NMR Data of **1** in DMSO-d_6

No.	^{13}C	^1H
1	137.75	
2	118.02	
3	155.88	
4	101.62	6.30 (1H, d, $J=1.7$ Hz)
5	156.82	
6	101.81	6.63 (1H, d, $J=1.7$ Hz)
1′	128.72	
2′	112.30	6.60 (overlapped)
3′	145.31	
4′	145.49	
5′	115.59	6.61 (1H, d, $J=8.2$ Hz)
6′	118.65	6.51 (1H, dd, $J=8.2, 1.7$ Hz)
1″	128.23	
2″	127.59	
3″	111.25	7.17 (1H, s)
4″	144.40	
5″	145.02	
6″	118.65	6.41 (1H, s)
1‴	139.44	
2‴, 6‴	103.97	6.08 (2H, d, $J=1.7$ Hz)
3‴, 5‴	158.32	
4‴	101.52	6.00 (1H, br s)
α	123.79	6.34 (1H, d, $J=16.1$ Hz)
β	128.36	6.74 (1H, d, $J=16.1$ Hz)
α'	126.74	6.49 (1H, d, $J=16.3$ Hz)
β'	125.14	6.59 (1H, d, $J=16.3$ Hz)

Table 2. Superoxide Anion Scavenging Activity of Compounds from *C. tibetica*[a]

Compounds	IC$_{50}$ (μM, $\pm$S.E.)
(+)-Catechin[b]	3.67±0.14
Tibeticanol (**1**)	1.33±0.01
Scirpusin B (**9**)	2.83±0.03
Komposinol A (**7**)	2.99±0.05
Piceatannol (**3**)	4.66±0.14
Scirpusin A (**8**)	4.68±0.14
Cassigarol G (**6**)	4.89±0.13
Cassigarol E (**5**)	11.49±0.18
	Activity at 100 μM (%)
Resveratrol (**2**)	50.5±1.7
Isorhapontigenin (**4**)	45.7±0.7
Syringaresinol (**12**)	55.1±0.3
Medicarpin (**11**)	No activity

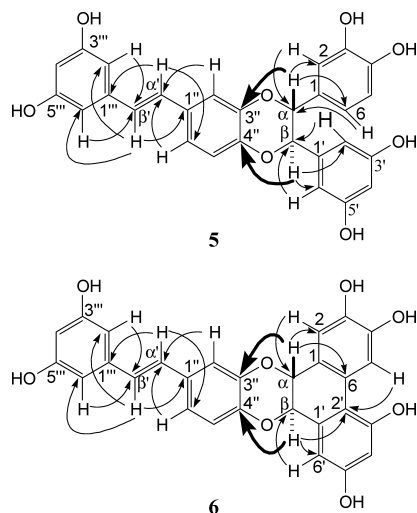*a*) $n=4$.   *b*) Positive control.

Fig. 2. Significant HMBC Correlations

Observed in the CT-HMBC spectra of **5** and **6**.

stilbene. It is well known that horse radish peroxidase (HRP) is a good oxidative enzyme that catalyzes the formation of C–O or C–C coupled products from phenolic substrates.[14—16] Since cassigarol G (**6**), a piceatannol dimer, was obtained in a low yield (1.5 mg), it was difficult to investigate its superoxide scavenging activity. In order to obtain an enough quantity of cassigarol G (**6**) for biological activity testing, phenol oxidative coupling reaction of monomeric stilbene piceatannol (**3**) was carried out with horse radish peroxidase (HRP) and hydrogen peroxide system. Two main products were isolated from the reaction mixture and identified to be cassigarol E (**5**) and cassigarol G (**6**) by NMR spectroscopy. C–C coupling product was not obtained from the reaction mixture. The results indicate that the reaction is initiated by the oxidation of the phenol group of catechol para to the olefinic double bond.

Superoxide anion scavenging activity of the isolated compounds was tested except for sativan (**10**), which was obtained in too low yield to submit for the assay. The results are summarized in Table 2. Tibeticanol (**1**) showed the strongest activity, which was followed by scirpusin B (**9**), komposinol A (**7**), piceatannol (**3**), scirpusin A (**8**), cassigarol G (**6**) and cassigarol E (**5**). The other compounds showed no significant effect in the bioassay system. The results indicate that the presence of catechol groups is essential for high activity and well conjugated structures also contribute to their antioxidant activity. This would be the reason why tibeticanol (**1**) showed the highest activity, since it possesses two catechol groups in a molecule and a well conjugated structure. Scirpusin B also possesses two catechol groups, but it lacks conjugated structure. This results in a slightly decreased activity. Piceatannol, scirpusin A and cassigarol G exhibit similar activity, since they possess only one catechol group. The structural difference between cassigarol G and cassigarol E is the C–C bond between C-6 and C-2′ in cassigarol G, which affords cassigarol G a well conjugated structure and results in a higher antioxidant activity than cassigarol E.

The stems of *C. tebetica* are used to treat atherosclerosis, hyperlipidemia, hypertension and arthritis which are regarded to be related to reactive oxygen species (ROS) and lipid peroxidation.[17] The antioxidant activity of the isolated stilbenoids of *C. tibetica* indicates that they are the active constituents for the treatment of diseases mentioned above.

Experimental

Plant Material The stems of *C. tibetica* were provided by Arura Ti-

betan Medicine Center. A voucher specimen (K031025A) is deposited in Kanebo Kampo Health Care Institute, Japan. The plant was identified at Arura Tibetan Medicine Center.

Extraction and Isolation The dried stems of *C. tibetica* (325 g) were cut into small pieces and extracted twice by refluxing with 70% acetone at 55 °C (2 h, 1 h) to give a 70% acetone extract (*ca.* 30 g). The extract was suspended in water, and then partitioned successively with hexane, chloroform and ethyl acetate, to obtain a hexane fraction (*ca.* 1 g), chloroform fraction (*ca.* 2.5 g), ethyl acetate fraction (*ca.* 18.5 g) and water fraction (*ca.* 8.0 g). The ethyl acetate fraction was further chromatographed on a Sephadex LH-20 column eluted with water/methanol (0—100%) to give 9 fractions. Fr. 3 (20—40% MeOH eluate) was submitted to silica gel chromatography and eluted with $CHCl_3$/MeOH to give syringaresinol (8.0 mg). Fr. 4 (60% MeOH eluate) was further isolated by silical gel and Sephadex LH-20 chromatography to give kompasinol A (149.2 mg). Fr. 5 (60% MeOH eluate) was subjected to silical gel, Sephadex LH-20, mid-pressure column chromatography and reversed-phase TLC to give resveratrol (73.5 mg), piceatannol (*ca.* 500 mg), isorhapontigenin (45.4 mg), sativan (2.0 mg), medicarpin (15.0 mg), respectively. Scirpusin A (5.0 mg) and B (25.4 mg), cassigarol E (*ca.* 30 mg), cassigarol G (1.5 mg) and tibeticanol (7.3 mg) were obtained from Fr. 6 and 7 (80% MeOH eluate).

Compound **1**: Amorphous brown powders; HR-FAB-MS *m/z*: Found 485.1261, Calcd for $C_{28}H_{22}O_8$–H: 485.1236; UV λ_{max} (MeOH) nm (log ε): 330 (4.44), 220 (4.52); ^{1}H-NMR (DMSO-d_6, 500 MHz) and ^{13}C-NMR (DMSO-d_6, 125 MHz), see Table 1.

HRP Catalyzed Oxidative Coupling of Piceatannol The reaction was performed by using the reported method.[14] A solution of piceatannol (244 mg) and HRP (100 unit) in acetone (70 ml) and water (28.5 ml) was treated with hydrogen peroxide (3%, 1.1 ml) at 25 °C and stirred for 1.5 h. The reaction mixture was extracted with ethyl acetate three times after the acetone was removed. The extracts were combined together and washed with water twice, dried with Na_2SO_4, and concentrated *in vacuo* to give a crude extract (220 mg). The crude extract was dissolved with a small amount of methanol which was then applied to a Sephadex LH-20 column (2×45 cm) chromatography, eluted with water/methanol (55—100%) to give 9 fractions. Fr. 4 (*ca.* 35 mg) from 60% methanol was further separated with reversed-phase TLC (developed with 60% MeOH) to give cassigarol E (*ca.* 20 mg); Fr. 8 (*ca.* 23 mg) from 70% methanol was further separated with reversed-phase TLC (developed with 70% MeOH) to give cassigarol G (*ca.* 13 mg).

Assay for Superoxide Anion Scavenging Activity Superoxide anion scavenging activity was tested according to the reported method[18] with some modifications. 50 mM Na_2CO_3 buffer (pH 10.2, 120 μl), 3.0 mM xanthine, 3.0 mM EDTA, 0.15% BSA and 0.75 mM NBT each 5.0 μl were added to the 96-well microtiter plate, successively. After 99.9% ethanol (5.0 μl) or the test sample (dissolved with 99.9% ethanol, 5.0 μl) were added to the above mixture, it was pre-incubated at 25 °C for 10 min. This was followed by the addition of 250 fold diluted xanthine oxidase (5.0 μl, 7.0×10^{-4} U) or Na_2CO_3 buffer (5.0 μl) to the mixture, and incubation at 25 °C for 20 min. The reaction was stopped by adding 6 mM $CuCl_2$ (5.0 μl), and the absorbance at 560 nm of the reaction mixture was measured. Superoxide anion scavenging activity was calculated by the following equation: scavenging activity=$[1-(A-A')/(B-B')]\times100$, and was given as the mean value of four experiments. A is the absorbance of the reaction mixture containing test sample and XOD; A′ is the absorbance of the reaction mixture containing test sample but without XOD which was replaced with 5.0 μl Na_2CO_3 buffer; B is the absorbance of the reaction mixture containing XOD without test sample which is replaced by 99.9% ethanol 5.0 μl; B′ is the absorbance of the reaction mixture without test sample and XOD which are replaced by 99.9% ethanol 5.0 μl and Na_2CO_3 buffer 5.0 μl.

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