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Anti-oxidative and Antibacterial Constituents from *Sedum hybridum*

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Abstract – Phytochemical studies on the whole extract of *Sedum hybridum* L., a Mongolian medicinal plant, has been undertaken to isolate active principles responsible for its anti-oxidative and antibacterial activities. Eighteen known compounds, *i.e.* (**1**) quercetin, (**2**) kaempferol, (**3**) herbacetin-8-O-β-*D*-xylopyranoside, (**4**) myricetin, (**5**) gossypetin-8-O-β-*D*-xylopyranoside, (**6**) gallic acid, (**7**) 2,4,6-tri-O-galloyl-*D*-glucopyranose, (**8**) 6-O-galloylarbutin, (**9**) myricetin-3-O-α-*L*-arabinofuranoside, (**10**) quercetin-3-O-α-*L*-arabinofuranoside, (**11**) caffeic acid, (**12**) ethylgallate, (**13**) (-) epigallocatechin-3-O-gallate, (**14**) palmitic acid, (**15**) stearic acid, (**16**) stearic acid ethyl ether, (**17**) β-sitosterol and (**18**) β-sitosteryl-O-β-*D*-glucopyranose have been isolated and their molecular structures identified by spectroscopic analysis. Thirteen substances including seven flavonol components (**1, 2, 3, 4, 5, 9** and **10**), five gallic acid derivatives (**6, 7, 8, 12** and **13**) and caffeic acid (**11**) exhibited significant, dose-dependent, DPPH radical scavenging activity. Galloyl esters **12** and **13** were revealed to be main active principles for the antibacterial property of the extract of *Sedum hybridum* L.
Key words – *Sedum hybridum*, Crassulaceae, phenolics, anti-oxidative, antibacterial

Introduction

Sedum hybridum L. (Crassulaceae), a stonecrop, succulent leaf plant, is one of the 5 species of Sedum grown throughout the Mongolian territory (Grubov, 1982).

The aerial part of *Sedum hybridum* L. is usually used in traditional medicine for the treatment of various infections and inflammation-associated diseases, such as diarrhea, dysentery, sepsis and blood vessel disease as well as thyroid disease. This plant specieshas been used as hemostatic, antiseptic, antibacterial, and anti-fever treatments and as a tonic for the central nervous system (Khaidav *et al*., 1969; Khaidav *et al*., 1978; Ligaa, 2006; Budachev, 2009). Moreover, fresh juice of the leaves is used for healing of wounds, ulcers, sores and other external injuries (Sviridinov, 1978).

However, in spite of various pharmacological effects of the species, only one brief report is available on the phytochemical constituents of *S. hybridum.*

During the course of searching for anti-oxidative or anti-infective natural products from the Mongolian flora, we found that the whole plant extract of *S. hybridum* exhibited an excellent anti-oxidative effect evaluated by a stable DPPH radical scavenging assay *in vitro*. Moreover, it has also demonstrated a significant anti-proliferative effect upon the growth of several bacterial strains, such as *Micrococcus luteus, Escherichia coli, Staphylococcus aureus, Enterococcus faecalis* and *Pseudomonas aeruginosa.*

For the purpose of identifying active constituents responsible for anti-oxidative or anti-infective activity, extensive phytochemical studies have been done on the plant extract of *S. hybridum,* which finally led to isolation of the eighteen compounds.

Experimental

General – TLC was carried out on pre-coated silica gel 60 F_{254} plates (Merck, Darmstadt, Germany) and spots were detected under UV radiation (254 nm) by spraying with 1% methanolic diphenylboric acid-β-ethylamino ester (NP), and 5% ethanolic polyethylene glycol (PEG). For column chromatography (CC) Sephadex LH-20 (25 - 100 μm, Pharmacia, Uppsala, Sweden), MCI gel – CHP-20P (75 - 150 μm, Mitsubishi Chemical Corporation, Japan), Sepra C18-E (50 μm, 65A), and Silica gel 60 (40 - 60 μm) were used. UV spectroscopic analysis was done on a spectrophotometer (Shimadzu, UV-160, Japan)

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using 5% $AlCl_3$, 1 N HCl, CH_3COONa and H_3BO_3 diagnostic reagents. NMR spectra in DMSO-d_6 including ^1H and ^{13}C NMR were recorded on Bruker AM-300 and 75 MHz instruments, respectively.

Plant material – Aerial parts of *Sedum hybridum* L. were collected from the mountain chain of the Gobi-Altai province in June 2006. The systematic identification of this species was authenticated by Prof. Jamsran Ts., Department of Botany, National University of Mongolia. A voucher specimen (NUM-06-024) was deposited in the herbarium of the Natural Product Chemistry Laboratory of the Institute of Chemistry and Chemical Technology, Mongolian Academy of Sciences.

Extraction and fractionation – Air-dried and chopped aerial parts (1,450 g) of *S. hybridum* were exhaustively extracted with 14 L of 80% ethanol at room temperature. The ethanol extract was filtered and concentrated under vacuum at 40 ºC to give 300 g of thick residue which was suspended in water and fractionated successively with an equal volume of dichloromethane (DCM), ethylacetate (EA) and *n*-butanol (*n*-BuOH), to yield 2.05 g of a DCM soluble fraction, 31.5 g of an EA soluble fraction and 126.0 g of an *n*- BuOH soluble fraction.

Isolation and Identification – The EA fraction (28.0 g) was divided into ten subfractions (Fr. I – Fr. X) by Sephadex LH 20 CC, eluting with a mixture of 50 % DCM in MeOH – 1 : 1 as: FrI-1.61 g, FrII-1.35 g, FrIII-5.67 g, FrIV-1.99 g, FrV-6.0 g, FrVI-1.51 g, FrVII-1.83 g, FrVIII-4.57 g, FrIX-0.49 g and FrX-48.9 mg, respectively.

Fractions FrIII – FrVIII were purified by repeated CC with MCI gel CHP 20P, Sephadex LH 20, ODS and Silica gel, which finally gave compounds 1-18 in the following amounts: (**1**) 39.7 mg, (**2**) 277.5 mg, (**3**) 64.4 mg, (**4**) 5 mg, (**5**) 33.7 mg, (**6**) 234.5 mg, (**7**) 60.6 mg, (**8**) 217.7 mg, (**9**) 26.2 mg, (**10**) 34.1 mg, (**11**) 22.3 mg (**12**) 226 mg, (**13**) 300.15 mg, (**14**) 67.8 mg, (**15**) 351.9 mg, (**16**) 51.6 mg, (**17**) 160 mg and (**18**) 133.9 mg.

DPPH radical scavenging activity – DPPH radical (2,2-diphenyl-2-picrylhydrazyl hydrate, Sigma-Aldrich Inc) scavenging activity of plant extracts and pure compounds was determined by spectrophotometric assay (Mensor *et al*., 1978). Crude extracts, fractions and pure compounds were dissolved in MeOH (1.0 mg/mL) and then diluted to final concentrations of 200, 100, 50, 25, 12.5 and 40, 20, 10, 5, 2.5, 1.25 μg/mL with MeOH. 1.5 mL of each sample was added into 1.5 mL of DPPH solution (6×10^{-5} M). The reaction mixture was shaken gently and allowed to react in the dark for 30 min at room temperature. The absorbance was then measured at 517 nm on a spectrophotometer (UV-160, Shimadzu, Japan) and the antioxidant activity (AA) was expressed in the percentage:

$$\grave{A}A\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\};$$

Methanol (1.5 mL) added to plant extract solution (1.5 mL) was used as a blank. DPPH solution (1.5 mL, 6×10^{-5} M) plus methanol (1.5 mL) was used as a control.

The IC_{50} value, defined as the amount of the sample which reduced the initial concentration of DPPH by 50%, was calculated from a linear regression of plots of test sample concentrations (μg/mL) against the mean inhibition in percentage. Rutin (Roth Inc, Germany) was used as a positive control. IC_{50} values were calculated using concentrations of tested plant extracts and the average percentage of the antioxidant activity from three separate tests.

Antibacterial activity – Antibacterial activity was tested against bacterial strains *Pseudomonas aeruginosa*, *Escherichia coli*, *Enterococcus faecalis*, *Micrococcus luteus* and *Staphylococcus aureus* by the disc diffusion method (Karaman *et al*., 2003). One hundred microliters of test microorganisms (10^6 colony forming units (CFU)/mL), grown in nutrient broth media for 24 h, were spread over the surface of meat peptone agar medium in 9 cm diameter Petri dishes. Filter paper discs (7 mm diameter) loaded with extracts (5000 μg/disc), fractions (5000 μg/disc) and pure compounds (1500, 1000, 750, 400, 300, 200, 40, 30, 20, 10 μg/disc) were placed on the surface of the Petri dishes. Petri dishes were incubated at 37 ºC for 24 h, and then diameters of inhibition zones were measured in millimeters. Kanamycin was used as a standard antibiotic.

Statistical Analysis – All data are expressed as mean ± standard deviation (S.D.).

Results and Discussion

The EtOH extract and each solvent fraction obtained from the extract by a serial solvent partition process, including the final water residue as well as the aqueous extract, were assayed for free radical scavenging activity.

The concentration of extract that caused a 50% decrease in DPPH radical scavenging activity (IC_{50}, in μg/mL) was calculated to be 31.93 ± 0.65 μg/mL, when that of the standard reference, rutin, was calculated as 22.66 ± 0.29 μg/mL. However, EA and *n*-BuOH fractions exhibited better radical scavenging activity with IC_{50} values of 27.11 ± 0.58 μg/mL and 26.14 ± 0.31 μg/mL, respectively (Fig. 1).

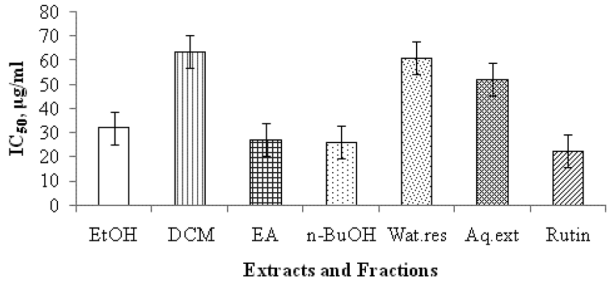
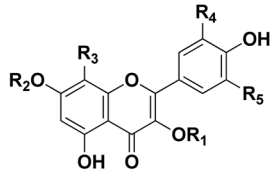


Fig. 1. IC_{50} (μg/mL) values of extracts and fractions for DPPH radical scavenging.



	R_1	R_2	R_3	R_4	R_5
1	H	H	H	OH	H
2	H	H	H	H	H
3	H	H	Xyl	H	H
4	H	H	H	OH	OH
5	H	H	Xyl	H	OH
9	Ara	H	H	OH	OH
10	Ara	H	H	OH	H

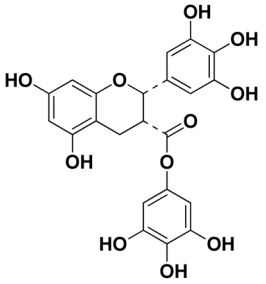


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Fig. 2. Structures of isolated flavonoids.

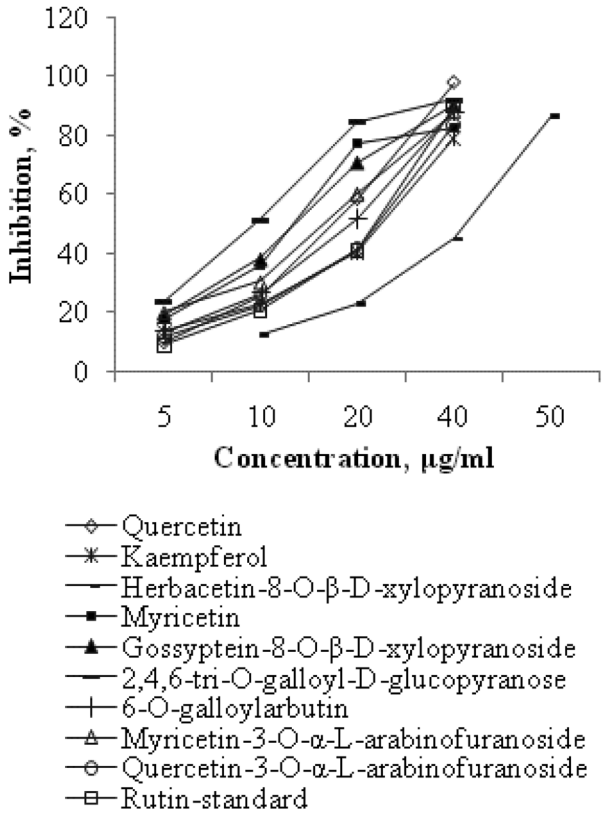

Fig. 3. DPPH radical scavenging activity of isolated compounds.

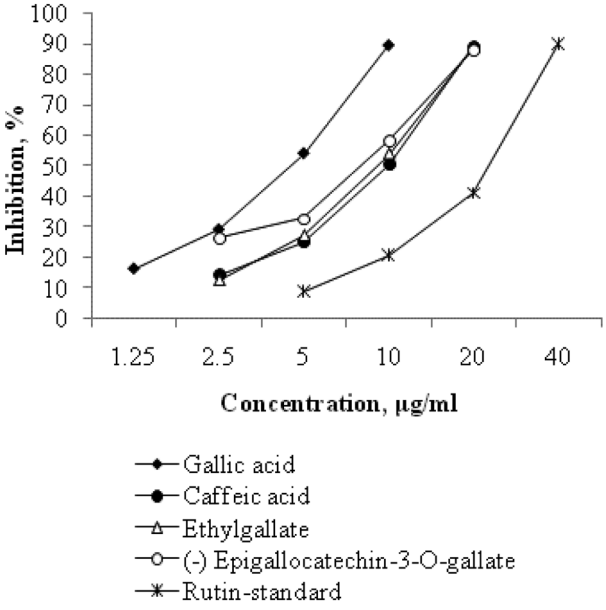

Fig. 4. DPPH scavenging activity of isolated compounds.

Previously we reported that the EtOH extract of *S. hybridum*, in particular, the EA fraction derived from the EtOH extract, exhibited a strong antibacterial activity against gram positive strains, *M. luteus* and *S. aureus*, while DCM and *n*-BuOH fractions were only weakly active. Moreover, all tested extracts and fractions exhibited no activity against gram-negative bacterial strains *E. coli* and *P. aeruginosa* (Enkhmaa *et al*., 2008).

Referring to results of antioxidative and antimicrobial activities of the extract and fractions, the phytochemical study was focused on the EA fraction. By repeated column chromatography of EA fraction of the plant extract with various packing materials, eighteen kinds of known compounds were isolated and identified as (**1**) quercetin (Markham *et al*., 1978; Harborne, 1994), (**2**) kaempferol (Markham *et al*., 1978; Harborne, 1994), (**3**) herbacetin-8-O-β-*D*-xylopyranoside (Kurkin *et al*., 1984; Tuong *et al*., 2007), (**4**) myricetin (Agrawal *et al*., 1981), (**5**)gossypetin-8-O-β-*D*-xylopyranoside (Tuong *et al*., 2007), (**6**) gallic acid (Sato *et al*., 1997), (**7**) 2,4,6-tri-O-galloyl-*D*-glucopyranose (Sato *et al*., 1997), (**8**) 6-O-galloylarbutin (Chen *et al*., 1987), (**9**) myricetin-3-O-α-*L*-arabinofuranoside (Yasikawa *et al*., 1990), (**10**) quercetin-3-O-α-*L*-arabonofuranoside (Markham *et al*., 1978; Harborne, 1994), (**11**) caffeic acid (Cui *et al*., 1990), (**12**) ethylgallate (Sato *et al*., 1997), (**13**) (–) epigallocatechin-

3-O-gallate (Sakar *et al*., 1993), (**14**) palmitic acid (Bus *et al*., 1976; Gunstone *et al*., 1976; Knothe *et al*., 2004), (**15**) stearic acid (Bus *et al*., 1976; Gunstone *et al*., 1976; Knothe *et al*., 2004), (**16**) stearic acid ethyl ether (Bus *et al*., 1976; Gunstone *et al*., 1976; Knothe *et al*., 2004), (**17**) β-sitosterol (Ahmad *et al*., 2010) and (**18**) β-sitosteryl-O-β-*D*-glucopyranose (Usuki et al., 2008), by comparison of their physico-chemical and spectroscopic data with those reported in the literature (Fig. 2).

Among the isolated compounds, compound **3, 4, 5, 7, 8, 9, 10, 12, 13, 14, 15, 16, 18** have been isolated from *S. hybridum* for the first time. Compounds **2, 6, 8, 9, 12** and **13** were isolated in a large amount, then regarded as main components of *S. hybridum.*

Thirteen substances including seven flavonol components (**1**, **2**, **3**, **4**, **5**, **9** and **10**), five gallic acid derivatives (**6**, **7**, **8**, **12** and **13)** and caffeic acid (**11**) exhibited a significant DPPH radical scavenging activity in a dose dependent

Table 1. Antibacterial activities of pure compounds

Samples	Dose, μg/disc	Inhibition zone, mm				
		E.coli	P. aeruginosa	S.aureus	E.faecalis	M.luteus
1	200	–	–		–	–
	300	–	–		–	–
	400	–	–	8.6	–	–
	750	–	–	+++	–	9.1
	1000	–	–	+++	–	9.5
2	200	–	–	8.6	–	–
	300	–	–	9.2	–	
	400	–	–	9.6	–	
	1500	–	–	+++	–	+++
4	200	–	–		–	–
	300	–	–	9.0	–	–
	400	–	–	9.4	–	–
6	200	–	–	10.0	–	–
	300	–	–	10.5	–	–
	400	–	–	10.5	–	–
	1500	–	–	+++	–	–
12	200	9.2	–	–	9.2	10 μg- (–)
	300	10.9	–		10.9	20 μg- 10.3
	400	13.5	–	9.8	13.2	30 μg- 13.2
	1000	+++	faint	+++	+++	40 μg- 15.8
13	200	–	–	–	–	9.1
	300	–	–	–	–	10.4
	400	–	–	–	–	12.1
	1000	–	–	12.2	–	+++
	1500	–	–	13.9	9.9	+++
14	200	–	–	9.1	–	–
	300	–	–	10.0	–	–
	400	–	–	10.5	–	–
15	750	–	–	-	–	9.4
	1000	–	–	9.0	–	9.7
	1500	–	–	9.8	–	10.1
16	750	–	–	9.1	–	–
	1000	–	–	9.2	–	–
	1500	–	–	9.7	–	–
Kanamycin	Control	13.7	10.4	18.0	11.0	16.0

Note: (–) – less active (less than 7.5 mm inhibition zone); (⊥) – active (7.5-8.5 mm); (+++) – very active (higher than 13.5 mm).

manner (Fig. 3 and Fig. 4). In spite of the difference in evaluation methods, the DPPH radical scavenging activity of these compounds was in accord with previous published results (Rice-Evans *et al*., 1996; Pietta, 2000; Haslam, 1996).

Among the isolated components, nine compounds **1, 2, 4**, **6**, **12**, **13**, **14**, **15** and **16** exhibited an inhibition upon the growth of more than one bacterial strain (Table 1).

The compound **12** exhibited a prominent antibacterial activity against the growth of all tested bacterial stains. In particular, it strongly inhibited the growth of *M. Luteus, E. Faecalis, E. coli* and *S. aureus*, respectively. However, it showed a poor inhibitory effect upon the growth of gram negative *P. aeruginosa.* at the concentration of 1000 μg/disc. Compound **6** showed moderate inhibition on the growth of *S. aureus* at the the concentration of 200 μg/disc. Compound **13** exhibited an inhibitory activity against the growth of all gram positive bacterial strains. It is notable that this compound remarkably inhibited the growth of *M. luteus* at the concentration of 200 μg/disc to give 9.1 mm of inhibitory zone, while it showed moderate inhibition on the growth of *S. aureus* at the concentration of 1000 μg/disc and *E. faecalis* at the concentration of 1500 μg/disc. These results supported the previous report that some naturally occurring tannins such as **6**, **12** and **13** are effective antibacterial agents of natural origins which have the ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, etc. (Sato *et al*., 1997; Cowan, 1999).

Flavonol components, **1**, **2** and **4** exhibited good antibacterial activity only against the growth of *S. aureus* at the concentration of 200 μg/disc. The free fatty acid, compound **14,** produced an inhibition zone of 9.1 mm at a dose of 200 μg/disc against *S. aureus*, while compounds **15** and **16** were active only at the highest doses, 750 and 1000 μg/disc, respectively. Moreover, compound **15** was active against *M. luteus* too.

However, these results indicated that galloyl esters (compounds **12** and **13**) were the main active principles for the antibacterial property of the extract.

In conclusion, the excellent antibacterial activity and anti-oxidative effect of the extracts from *Sedum hybridum* might be due to the high content of phenolic compounds such as flavonol and galloyl derivatives in *Sedum hybridum*.

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