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Antioxidant Activity of Cholesterol Derived from Silkworm Pupae

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Abstract – To search of more selective vasculogenic relaxation activity, the antioxidant activity of silkworm male pupae was determined by measuring its radical scavenging effect on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals, and anticoagulant activity of them was measured clotting time in both activated partial thromboplastin time (aPTT). Because, most of cGMP-enhancing agent such as, sildenafil, promotes thrombin-induced platelet aggregation, developed unexplained thrombic conditions including heart attack. To search more suitable and safe drug for vasculogenic relaxation, we purified silkworm pupae male extract. The ethyl acetate extract of silkworm male pupae showed strong scavenging activity in both DPPH and aPTT anticoagulant activity. The antioxidant activity potential of the individual fraction was in order of ethyl acetate > *n*-butanol > chloroform > *n*-hexane. The ethyl acetate soluble fraction exhibiting strong anti-oxidant and anticoagulant activity was further purified by repeated silica gel and Sephadex LH-20 column chromatography. Cholesterol was isolated as one of the active principles from ethyl acetate fraction, together with, minor portion, β-sitosterol.
Keywords – Silkworm pupae, cholesterol, antioxidant, β-sitosterol

Introduction

In Oriental Asia, male silkworm cocoon extract has been known for its effectiveness in enhancing of male stamina and improving vitality. Its main ingredients are reported as protein (51%), fatty acid (29%), saccharide (2%), cholesterol (3%), chitin and Vitamins A, B2 and D (Cui et al., 2002). However, its active component that affected endothelial vasorelaxation was not known until now. In this study, we purified the active vasorelaxation substances in male silkworm pupae by organic solvent extraction. In case of mulberry leaves, flavonoids have free radical scavenging activity using the α-diphenyl-β-picrylhydrazyl (DPPH) radical, and of these, quercetin-3-*O*-β-D-glucopyranosyl-(1->6)-β-D-glucopyranoside and quercetin were found to possess antioxidant activities (Kim *et al*., 1999). But, the antioxidant component of silkworm was uncertain yet, the main component responsible for the improvement in erectile dysfuction and its detailed mechanism remain uncertain. The ethyl acetate extract of silkworm pupae predominantly contains cholesterol, β-sitosterol, β-ecdysone and fatty acid such as myristic acid, palmitic acid, stearic acid, linoleic acid, and linolenic acid. Cholesterol, β-sitosterol and β-ecdysone in silkworm pupae have been shown to have testosterone-like effects (Zhou *et al*., 2006). Cholesterol is a substantial element to compose cell membrane, blood plasma and take part in primary role for inflammatory process in all animals (Hobson, 1935; Barter *et al*., 2004). Therefore because insects should molt their husks, it is also necessary. By dealkylation route, β-sitosterol, which function growth promoting, is transformed into cholesterol in silkworm, *Bombyx mori*. (Ikekawa *et al*., 1966.) Cholesterol, an important component in animals and insects, is caused to β-sitosterol which is essential for growth in silkworm, *Bombyx mori* as well as precursors of molting hormone,ecdysteroid.(Nagata *et al.*, 2006)

The mechanism of sildenafil citrate (Viagra®) and other similar drugs for the treatment of erectile dysfunction is well known. On the other hand, sildenafil enhances platelet activation in the presence of subthreshold concentrations of thrombin or vWF, and can cause platelet aggregation (Zhenyu *et al*., 2003). Food preparations (capsules) containing male silkworm pupae extract such as Nuegra® are currently commercially available in Korea and male silkworm pupae is considered a candidate nutraceutical agent or supplement to enhance masculine function.

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In this report, we now report the antioxidant evaluation of all extracts and isolated compounds from silkworm male pupae for its potential to scavenge stable DPPH free radicals and vasculogenic substances from silkworm pupae in order to develop a pharmaceutical candidate for treating vasculogenic impotence.

Experimental

Materials – Male silkworm pupae were reared and supplied by the Department of Agricultural Biology, National Institute of Agricultural Science and Technology, Korea. Fibrinogen (bovine plasma), thrombin and aPTT reagent were purchased from Sigma Co. (St. Louis, USA.).

Preparation of Test Solution – *Extraction and isolations of antioxidant substances*

The dried male silkworm pupae (500 g) were soaked and extracted three times with EtOH by ultrasonification for 30 min. The extracts obtained were dried on a rotary evaporation; the residue was suspended in water and successively extracted with *n*-hexane, chloroform, ethylacetate, *n*-butanol and H_2O in sequence. The EtOAc fraction showed strong scavenging activity against DPPH radical, and anticoagulant activity on aPTT test. Thus, the EtOAc (g) fraction was chromatographed on a silica gel column using CH_2Cl_2-MeOH (gradient) to give compound 1 and 2, respectively. Each sample (10 mg) was dissolved in 500 µL of PBS buffer (final concentration 0.5% ethanol or 0.5% DMSO) as a test solution.

Effects on coagulation systems – Human plasma from the Blood Bank of Seoul National University Hospital was used for measuring clotting time in both activated partial thromboplastin time (aPTT) and thrombin time (TT). The clotting time tests were performed on a Beckton Dickenson BBL Fibrosystem (Cockeysville, USA.). In brief, a mixture containing 40 µL of test solution and 80 µL of prewarmed plasma for one minute was incubated for three minutes under stirring, then dropped into 0.02 M calcium chloride solution at 37 °C When the clot was formed, aPTT was measured. For the measurement of TT, an equal volume of thrombin (10 U/mL) and test insect fractions were mixed and incubated at 37 °C for 5 minutes. The 50 µL of reaction mixture was added to 250 µL of pre-warmed fibrinogen and the clotting time was determined (Astrup and Mullertz, 1952).

DPPH radical scavenging effect – The DPPH radical scavenging effect was evaluated according to the method first employed by Blois (Blois, 1958). One hundred and sixty microliters of MeOH solution of varying sample concentrations (0.25 - 160 µg/mL) was added to 40 µL DPPH methanol solution (1.5×10^{-3} M). After mixing gently and leaving for 30 min at room temperature, the optical density was measured at 520 nm using a spectrophotometer. The antioxidant activity of each fraction and sample was expressed in terms of IC_{50} microgram per ml concentration required to inhibit DPPH radical formation by 50% and calculated from the log-dose inhibition curve (Jung *et al*., 2001).

Cholestrol and β-sitosterol identification of EI mass spectrometry – EI (electron ionization) mass spectroscopy analysis was performed using an Electron 70 eV DIP (Direct Inlet Probe) mode in National instrumentation center for environmental management of Seoul National University. All MS data were processed using in the mass databases by the webbook program (NIST, Gaithersburg, MD).

General Instruments – NMR spectra were obtained at 600 MHz (^{1}H) and 600 MHz (^{13}C) on a high-resolution spectrophotometer (Avance 600 FT, Bruker, Germany) in using TMS as in an internal standard. UV spectra were measured on a JASCO V-550 UV/Vis spectrophotometer and IR spectra using a Jasco FT/IR-3300 spectrophotmeter on KBr plate, and melting points were determined on a Büchi B-540 melting point apparatus.

Fat acid analysis – Silkworm male pupae lipids to the method of Kates (1986), isolated total lipids of silkworm male pupae were esterified and the methyl ester of fatty acid concentration was measured by a gas chromatograph (Star 3600, Varaian Inc., Palo Alto, USA) equipped with a flame ionization detector (FID) and HP-FFAP capillary column (Nitroterephthalic acid modified polyethylene glycol, 30 m × 0.25 mm, 0.25 µm, USA).

Results and Discussion

Purification – The total EtOH extract of male silkworm pupae was partitioned into *n*- hexane, $CHCl_3$, EtOAc, *n*-BuOH, and H_2O fractions. To identify the active principles, we evaluated the antioxidant activity (DPPH) and the platelet aggregation inhibiting activity (aPTT) of these organic solvent soluble fractions.

Radical scavenging effect of the ethanol extract and their fractions of silkworm pupae on DPPH radical – Active oxygen species such as superoxide radicals, hydrogen peroxide and hydrogen radicals have been recognized as the principle agent responsible for the deterioration of polyunsaturated fatty acids, or lipid containing foods when supplied with electrons or hydrogen ions (Slater *et al*., 1987). The DPPH radical

Table 1. Antioxidant activity of extracts derived from silkworm male pupae on DPPH

Extract	DPPH[a]	Extract	Fraction	DPPH[a]
n-Hexan	1941.8	EtOAC	2	0.39
$CHCl_3$	>1000		3-1	1.08
EtOAC	29.64		3-2	0.57
n-BuOH	1298.3		4-1	4.14
H_2O	17.07		4-2	3.70
L-Ascorbic acid	1.37		5	2.12
Pyrogallol	0.26			

DPPH[a]: DPPH free radical scavenging activity (IC_{50}: μg/ml).

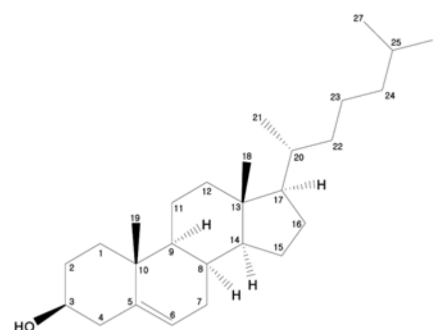
Table 2. Blood anticoagulant activity of ethyl acetate fractions derived from silkworm male pupae

Fraction	Thrombin Time (sec.)	aPTT* (sec.)	Fraction	aPTT* (sec.)
Control (PBS)	68	112		
1	78	137.8	2-1	125.0
2	208	999 <	2-2	167.0
3-1	88	96	2-3	136.0
3-2	62	124	2-4	160.5
3-3	104	136.5	2-5	176.0
3-4	98	147.2	2-5-1	152.0
4-1	65	115.7	2-5-1-1	120.5
4-2	56	133	2-6	77.5
4-3	81.5	149		
4-4	52.5	135		
5	53	138		
6	38.5	119		
7	35	93		

* aPTT, activated partial thrombopastin time
[1)] A mixture containing 40 μL of sample (1 μg) solution and 80 μL of prewarmed plasma for one minute was incubated for three minutes under stirring, then dropped into 0.02 M calcium chloride solution at 37 °C When the clot was formed, aPTT was measured. Values represent the mean and standard deviation of triplicate experiment.

scavenging effect for the ethanol extract and their fractions are shown Table 1. The ethyl acetate fraction (IC_{50}: 29.64 μg/mL), especially ethyl acetate (EtOAc) fraction 2 (IC_{50}: 0.39 μg/mL) displayed markedly increased DPPH free radical scavenging activity as shown in Table 1.

Effects of the anticoagulant activity on the ethanol extract and their fractions of silkworm pupae – The EtOAc fraction showed strong scavenging activity against DPPH radical, and furthermore, anticoagulant activity on aPTT test (Table 2). The EtOAc fraction, especially EtOAc fraction 2 (aPTT >200 sec) and EtOAc fraction 2-5 (aPTT >176 sec) displayed markedly increased blood anticoagulant activity as shown in Table 2.



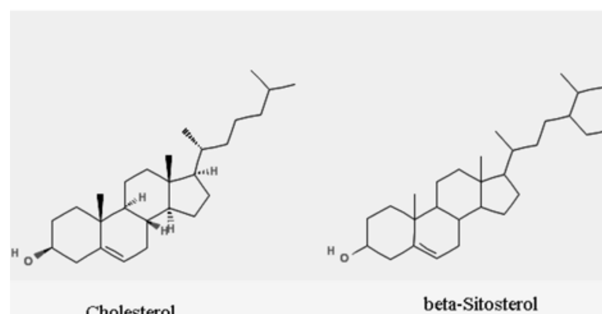


Fig. 1. Structures of compounds 1-2.

Thus, the EtOAc (g) fraction was chromatographed on a silica gel column using CH_2Cl_2-MeOH (gradient) to give compound **1** (cholesterol) and **2** (β-sistosterol), respectively. The chemical structures of these compounds are shown in Fig. 1.

Compound 1 – White powder, EI-MS *m*/*z*: 386 $[M]^+$, (Fig. 1). The molecular formula of **1** was determined as cholesterol ($C_{27}H_{46}O$) on the basis of EI-MS and ^{13}C-NMR data.

^1H-NMR ($CDC1_3$, 600 MHz) δ: 5.35 (1H, t, *J* = 2.51 Hz, H-6), 3.52 (1H, ddt, *J* = 5.41 Hz, H-3), 2.29 (2H, ddtt, *J* = 4.89 Hz, H-4), 2.00 (2H, t, *J* = 3.74 Hz, H-12), 1.96 (2H, d, *J* = 2.50 Hz, H-7), 1.85 (2H, t, *J* = 3.75 Hz, H-1), 1.84 (2H, s, H-2), 1.83 (2H, t, *J* = 4.51 Hz, H-25), 1.57 (2H, dd, *J* = 6.37 Hz, H-11), 1.53 (2H, d, *J* = 3.96 Hz, H-15), 1.51 (2H, dt, *J* = 4.80 Hz, H-23), 1.47 (1H, dt, *J* = 4.39 Hz, H-8), 1.15 (2H, t, *J* = 3.57 Hz, H-16, H-22, H-24), 1.10 (1H, dd, *J* = 7.17 Hz, H-17), 1.01 (3H, s, H-19, 1H, s, H-20), 0.99 (1H, d, *J* = 6.67 Hz, H-14), 0.94 (1H, d, *J* = 4.94 Hz, H-9), 0.93 (3H, d, *J* = 4.28 Hz, H-21), 0.86 (3H, dd, *J* = 3.08 Hz, H-26, H-27), 0.68 (3H, s, H-18)

^{13}C-NMR ($CDC1_3$, 150 MHz) δ: 140.97 (C-5), 121.91 (C-6), 71.99 (C-3), 56.98 (C-14), 56.37 (C-17), 50.35 (C-9), 42.53 (C-4), 42.49 (C-13), 39.99 (C-12), 39.73 (C-24), 37.47 (C-1), 36.71 (C-10), 36.40 (C-22), 35.99 (C-20), 32.12 (C-7, C-8), 31.85 (C-2), 28.44 (C-23), 28.22 (C-25), 24.50 (C-15), 24.04 (C-16), 23.02 (C-27), 22.77 (C-

26), 21.30 (C-11), 19.60 (C-19), 18.93 (C-21), 12.07 (C-18).

Compound 2 – EI-MS *m/z:* 414 $[M]^+$, (Fig. 2). The molecular formula of **2** was determined as β-sistosterol ($C_{29}H_{50}O$) on the basis of EI-MS and ^{13}C-NMR data.

^{1}H-NMR ($CDC1_3$, 600 MHz) δ: 5.35 (1H, t, J = 2.51 Hz, H-6), 3.52 (1H, ddt, J = 5.41 Hz, H-3), 2.29 (2H, ddtt, J = 4.89 Hz, H-4), 2.00 (2H, t, J = 3.74 Hz, H-12), 1.96 (2H, d, J = 2.50 Hz, H-7), 1.85 (2H, t, J = 3.75 Hz, H-1), 1.84 (2H, s, H-2), 1.57 (2H, dd, J = 6.37 Hz, H-11), 1.53 (2H, d, J = 3.96 Hz, H-15), 1.47 (1H, dt, J = 4.39 Hz, H-8), 1.34 (1H, t, J = 8.49 Hz, H-26), 1.32 (2H, d, J = 4.10 Hz, H-25), 1.26 (2H, d, J = 8.49 Hz, H-23), 1.17 (3H, t, J = 4.34 Hz, H-27), 1.15 (2H, t, J = 3.57 Hz, H-16), 1.15 (2H, d, J = 3.57 Hz, H-22), 1.08(3H, s, H-27), 1.10 (1H, dd, J = 9.49 Hz, H-17), 1.06 (1.01, s, H-28), 1.01 (3H, s, H-19), 1.01 (1H, s, H-20), 0.99 (1H, d, J = 6.67 Hz, H-14), 0.93 (1H, d, J = 4.94 Hz, H-9), 0.93 (3H, s, H-21), 0.84 (3H, d, J = 2.17 Hz, H-29), 0.68 (3H, s, H-18).

^{13}C-NMR ($CDC1_3$, 150 MHz) δ: 140.97 (C-5), 121.91 (C-6), 71.99 (C-3), 56.98 (C-14), 56.37 (C-17), 50.35 (C-9), 46.04 (C-24), 42.53 (C-4), 42.49 (C-13), 39.99 (C-12), 37.47 (C-1), 36.71 (C-10), 36.40 (C-22), 36.35 (C-26), 35.99 (C-20), 32.12 (C-7, C-8), 31.85 (C-2), 29.87 (C-26), 24.50 (C-15), 24.04 (C-16), 23.28 (C-25), 21.30 (C-11), 20.03 (C-27), 19.60 (C-19), 19.25 (C-28), 18.93 (C-21), 12.19 (C-29), 12.07 (C-18).

Fatty acid composition analysis of silkworm pupae - Fatty acid composition in ethyl acetate extract according to silkworm pupae stage and sex displayed markedly unsaturated fatty acid composition (male pupae 69%,

Table 3. Fatty acid composition in ethyl acetate extract according to silkworm pupae stage and sex

	Female	M11*	M12	M13	M14	M15	M16	M17
Myristic acid	0.16	0.19	0.18	0.03	0.18	0.17	0.18	0.19
Palmitic acid	19.65	25.20	25.73	25.53	24.66	25.70	25.43	25.19
Palmitoleic acid (ω-7)	1.55	1.36	1.33	1.27	1.29	1.45	1.39	1.38
Stearic acid	6.95	5.04	5.05	5.08	5.03	4.78	4.83	4.98
Oleic acid (ω-9)	39.13	25.89	25.68	25.55	26.04	25.78	25.29	26.57
Oleic acid (ω-7)	0.03	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Linoleic acid (ω-6)	5.18	7.64	7.40	7.63	7.62	7.40	7.65	7.64
γ-Linoleic acid (ω-6)	0.24	0.18	0.15	0.14	0.18	0.15	0.17	0.15
Linoleic acid (ω-3)	26.09	33.81	33.77	34.00	34.21	33.97	34.40	33.29
Eicosenoic acid (ω-9)	0.37	0.22	0.23	0.22	0.23	0.24	0.23	0.22
Eicosadienoic acid (ω-6)	0.05	0.03	0.06	0.01	0.08	0.03	0.03	0.03
Eicosatrienoic acid (ω-6)	0.09	0.07	0.07	0.11	0.07	0.06	0.05	0.06
Arachidonic acid	0.14	0.11	0.10	0.12	0.11	0.06	0.12	0.06
Eicosapentaenoic acid (ω-3) (EPA)	0.08	0.13	0.13	0.18	0.14	0.09	0.14	0.10
Docosatetraenoic acid	0.09	0.05	0.06	0.06	0.06	0.06	0.05	0.06
Docosapentaenoic acid	0.06	0.03	0.02	0.01	0.04	0.02	0.02	0.04
Docosahexaenoic acid (DHA)	0.12	0.05	0.04	0.05	0.04	0.05	0.04	0.04
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Saturated Fatty Acid	26.76	30.43	30.96	30.64	29.87	30.65	30.44	30.35
Unsaturated Fatty Acid	73.24	69.57	69.04	69.36	70.13	69.35	69.56	69.65
Mono unsaturated fatty acid	41.08	27.47	27.23	27.04	27.57	27.46	26.90	28.17
Poly unsaturated fatty acid	32.16	42.10	41.81	42.32	42.56	41.89	42.66	41.47
n3	26.35	34.03	33.97	34.24	34.44	34.13	34.59	33.47
n6	5.81	8.07	7.84	8.08	8.13	7.75	8.07	8.00
n6/n3	0.22	0.24	0.23	0.24	0.24	0.23	0.23	0.24
MUFA/SFA	1.54	0.90	0.88	0.88	0.92	0.90	0.88	0.93
PUFA/SFA	1.20	1.38	1.35	1.38	1.42	1.37	1.40	1.37

*M11~M17: The male silkworm pupae of 11~17 days after metamorphosis
n3 (ω-3): fatty acids which have the first unsaturated bond in the third position from the omega carbon
n6 (ω-6): fatty acids which have the first unsaturated bond in the sixth position from the omega carbon
MUFA: monounsaturated fatty acid; SFA: saturated fatty acid; PUFA: polyunsaturated fatty acid; SUFA: single unsaturated fatty acid

female pupae 73%) as shown in Table 3. The fatty acid composition ratio of silkworm pupae, especially steraric acid (4.8~7.0%) as a saturated fatty acid was different from compared to another report (29.3%, Kang *et al*., 2006), but consistent to a cricket (*Gryllus bimaculatus*) fatty acid data (8.8%, Ahn *et al*., 2000).

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