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Sedative Activity and Its Active Components of Zizyphi fructus

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Abstract □ Sedative activity of Zizyphi fructus was evaluated by potentiation of hexobarbital-induced hypnosis test and its active principles have been characterized as nornuciferine and lysicamine. A new cyclopeptide alkaloid, daechucyclopeptide-1 was isolated together with zizyphusine.

Keywords □ Zizyphi fructus, *Zizyphus jujuba* var. *inermis*, Rhamnaceae, sedative, active principles, nornuciferine, lysicamine, zizyphusine, cyclopeptide alkaloid, daechucyclopeptide-1.

Zizyphi fructus, the fruits of *Zizyphus jujuba* Miller var. *inermis* Rehder has been frequently prescribed by Chinese medical practioners to treat chronic bronchitis, consumption and blood diseases or as analeptic, expectorant, sedative and a taste modifier. Chemical constituents have been reported to contain sugars[1], triterpenoids[2,3], saponins[4], glycosides[5], flavonoids[5], c-AMP[6], c-GMP[7] and ethyl fructofuranoside.[8] In connection with our studies on sedative and/or tranquilizer from Zizyphus species we evaluated sedative activity and characterized its active alkaloidal components.

The MeOH extract of Zizyphi fructus was fractionated (see experimental method) and evaluated the sedative activity of each fractions by prolongation of hexobarbital-induced sleeping time in mice. As summarized in Table I, both ether soluble alkaloid and BuOH fraction showed strong activity in a dose dependent manner.

Specific activity of Et_2O alkaloid fr. was higher than that of BuOH fr. Column chromatography of the alkaloid fraction, followed by preparative TLC yielded four alkaloids. A new pyrrolidine alkaloid, daechualkaloid-A has been already reported.[9]

Daechualkaloid-C (lysicamine) and -E (nornuciferine) showed sedative activity (Table II). Since zizyphusine isolated from BuOH fr. did not show sedative activity[10], it seemed that most of sedative activity of Zizyphi fructus was contributed from two aporphine alkaloids, lysicamine and nornuciferine.

Daechualkaloid-C, yellow needles, mp 212°C, $C_{18}H_{13}NO_3$: M^+ m/z 291 showed a strong absorption at 1675 cm^{-1} in IR spectrum which indicated the presence of conjugation of a carbonyl group with two aromatic rings. The UV spectrum with λ_{max} 235, 271, 308, and 400 nm indicated that it possessed a 7-oxodibenzo[de,g] quinoline skeleton.[11] The ^{1}H-NMR spectrum displayed characteristic peaks for two methoxy groups at δ 4.10, 4.02, AB quartet aromatic protons at δ 7.79 (1H, J = 5.2 Hz, 4 -H), 8.91 (1H, J = 5.2 Hz, 5 -H) and five aromatic protons at δ 7.22 (1H,s, 3 -H), 7.55-7.78 (2H, m, 9 -H, 10 -H), 8.58 (1H, dd, J = 7.6, 2 Hz, 8 -H) and 9.17 (1H,d J = 8 Hz, 11-H). On the basis that spectral data including mass spectrum were similar to those of lysicamine[12], it is reasonable to conclude that daechualkaloid-C is identical with lysicamine.

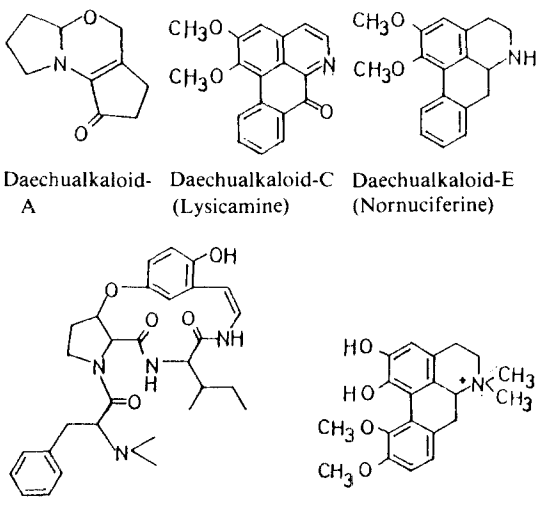

Fig. 1. Structures of alkaloids from Zizyphi fructus.

Table I. Effect of fractions of Zizyphi fructus on hexobarbital-induced sleeping time

control	Et_2O Alkaloid fr.			control	BuOH fr.		H_2O fr.
	50 mg	10 mg	3 mg/kg		1000 mg	300 mg	1000 mg/kg
22.3 ± 8.9	34.4 ± 10.3	32.3 ± 9.7		16.8 ± 5.7	33.4 ± 12.9	23.3 ± 9.7	
14.8 ± 7.4		22.2 ± 14.2	18.4 ± 10.5	18.0 ± 6.3	28.6 ± 13.0		19.0 ± 5.5

Samples in saline were orally administered 60 min. before hexobarbital sodium 50 mg/kg i.p. injection, Time in min. mean ± S.E., n = 6-7, 22-24 g mice

Table II. Effect of lysicamine and nornuciferine on hexobarbital-induced sleeping time

control	Lysicamine		Nornuciferine
	1 mg/kg	3 mg/kg	3 mg/kg
29.5 ± 11.1	34.3 ± 18.5	44.7 ± 15.4	46.4 ± 17.8

Samples in 1% CMC were administered by i.p. 30 min. before hexobarbital sodium 50 mg/kg i.p. injection. Time in min., mean ± S.E., n = 7, 24-28 g mice

Daechualkaloid-E, mp 154°C, showed an M^+ m/z 281, thus giving a possible molecular formula $C_{18}H_{19}NO_2$. The ^{1}H-NMR spectrum showed two methoxy groups at δ3.67, 3.88, one aromatic proton at δ6.64 (1H,s, 3 −H) and four aromatic protons at δ7.22-7.29 (3H,m, 8 −H, 9 −H, 10 −H) and 8.36 (1H,m, 11 −H) which were ascribed to the protons of the unsubstituted D ring of aporphine. ^{1}H-NMR data and mass spectrum were similar to nornuciferine.[13] Its acetate, mp 204°C, showed N-acetyl group(IR absorption at 1630 cm^{-1}, δ 2.18 (3H,s)). ^{1}H-NMR and mass spectrum were same with those of N-acetylnuciferine.[14] On the basis of the above data daechualkaloid-E is formulated as nornuciferine.

Daechucyclopeptide-1, mp 114°C, showed red color with Pauly's reagent indicating the presence of free phenolic hydroxyl group. ^{1}H-NMR spectrum was almost similar to that of daechuine-S_6[15] except lack of OCH_3 peak. Peaks at δ5.43 (1H, dt, J = 7, 2.8 Hz) and 4.38 (1H,d, J = 2.8 Hz) ascribed to β-and α-proton of hydroxyproline moiety respectively. Mass spectrum followed a typical fragmentation pattern for 13-membered peptide alkaloid.[16] M^+ m/z 534: $C_{30}H_{38}N_4O_5$, b m/z 443, r m/z 245, s m/z 219, t m/z 202 and u m/z 290 were 14 atomic mass units less than that of daechuine-S_6 which indicated 2,5-dihydroxystyrylamine unit was therein instead of 5-hydroxy-2-methoxy styrylamine unit. Base peak at m/z 148 reflected N,N-dimethylphenylalanine as terminal basic amino acid. The ions p m/z 209 and q m/z 181 indicated that β-hydroxyproline was bound on isoleucine and the ions r m/z 245, s m/z 219, t m/z 202 demonstrated that β-hydroxyproline was linked with 2,5-dihydroxystyrylamine. Ion u m/z 290 represented the direct linkage between 2,5-dihydroxystyrylamine and isoleucine; thus providing the structure of 13-membered ring system of daechucyclopeptide-1.

Alkaloid from BuOH fr. mp 185-187°C $[\alpha]_D$ + 312°C showed redish violet or red color with Pauly's reagent or $FeCl_3$, respectively, which was identified as zizyphusine by direct comparison with authentic sample, zizyphusine isolated from the seeds of *Zizyphus vulgaris* var. *spinosus*.

EXPERIMENTAL METHODS

Mps were determined on a Mitamura-Riken Heat Block Model MRK (uncorrected), UV spectra on a Gilford System 2600 spectrophotometer, IR spectra on a Perkin-Elmer 281 B spectrophotometer, and ^{1}H-NMR on a Varian FT-80A NMR spectrometer (80 MHz) with TMS as internal standard. Mass spectra were measured on a Hewlett-Packard 5985B GC/MS system (70 eV).

Animals and hexobarbital-induced sleeping time prolongation test

Male dd mice weighing 22-24 g were used and fed lab. chows and tap water. 6-7 mice in each group were injected with 50 mg/kg of hexobarbital sodium in saline at 60 minutes after oral administration or 30 minutes after intraperitoneal injection of the samples. Sleeping time was recorded from a loss of righting reflux to recovering of that.

Extraction and fractionation

Zizyphi fructus (12 kg) was extracted with boiling MeOH (60 *l*) for 4 hr two times. The MeOH extract (4.35 kg) was suspended in water (1 *l*) and extracted with Et_2O (1 *l* × 4), and then with n-BuOH (6 *l* × 4) to yield Et_2O fr. (87 g), BuOH fr. (297 g) and water fr. (3.97 kg).

The Et_2O fr. (85 g) was partioned between Et_2O and 5% HCl. The 5% HCl layer was washed with

Et_2O exhaustively and basified with c-NH_4OH to pH 9 and extracted with Et_2O to give alkaloid fr. (1 g).

Isolation of alkaloids

Et_2O soluble alkaloid fr. (0.98 g) was subjected to silica gel flash column chromatography and eluted with benzene: EtOAc (1:1) to give fr.1 (20 mg), fr.2 (200 mg) and fr.3 (330 mg), and then eluted with $CHCl_3$: MeOH (10:1) to give fr.4 (330 mg).

Daechualkaloid-A (155 mg) was isolated from fr.2 by preparative TLC (Hexane: EtOAc = 1:1), which has been reported.[9] Fr.3 (330 mg) was purified by silica gel flash column chromatography and preparative TLC ($CHCl_3$:EtOAc:MeOH = 50:10:1) to yield Rf 0.36, daechualkaloid-C which was crystalized from acetone (28 mg, yield 2.4×10^{-4} %), mp 212 °C, UV λ_{max}^{MeOH} nm (log ε) 235 (4.48), 271 (4.40), 308 (3.77), 400 (3.96). IR ν_{max}^{KBr} cm^{-1} 1675 (C = O). ^{1}H-NMR ($CDCl_3$, TMS) δ 4.02 (3H,s, 2-OCH_3), 4.10 (3H,s, 1 −OCH_3), 7.22 (1H,s, 3-H), 7.78-7.55 (2H,m, 9-H, 10-H), 7.79 (1H,d, J = 5.2 Hz, 4-H), 8.58 (1H, dd, J = 2.76 Hz, 8-H), 8.91 (1H,d, J = 5.2 Hz, 5-H), 9.17 (1H,d, J = 8 Hz, 11-H). MS, *m/z* (rel. int.) 291 (M^+, 44.5), 276 (M^+-15, 3.7), 261 (M^+-CH_2O, 1.7), 248 (100), 246 (1.3), 233 (14.8), 231 (0.5), 230 (0.5), 205 (11.2), 190 (10.4).

Fr. 4 (100 mg) was suspended in Et_2O (20 m*l*) and extracted with 5% NaOH (5 m*l* × 2). Et_2O layer gave 52 mg residue. 5% NaOH layer was titrated to pH 9 with 5% HCl and extracted with $CHCl_3$ (20 m*l* × 2) to yield 27 mg. $CHCl_3$ extract (27 mg) was purified by preparative TLC (Benzene:Acetone:MeOH = 30:25:4) to yield daechucyclopeptide-1 (5 mg, yield 4.1×10^{-5}%). mp 114 °C, ^{1}H-NMR ($CDCl_3$, TMS) δ 1.04-0.92 (6H, CH_3 × 2), 2.38 (6H,s, N-dimethyl), 4.38 (1H,d, J = 2.8 Hz, α-H of Hypro), 5.43 (1H, dt, J = 7, 2.8 Hz, β-H of Hypro), 5.75 (1H,d, J = 9 Hz, olefinic), 7.4-6.55 (10H,m aromatic H × 8, olefinic H × 1, NH), 8.4 (1H,d, J = 12 Hz, NH). MS, *m/z* (rel. int.) 534 (M^+, 0.2), 443 (M^+-91, 4.9), 290 (0.1), 245 (0.1), 219 (0.2), 209 (0.4), 202 (0.2), 181 (0.4), 151 (1.3), 148 (100), 96 (1.8), 68 (2.6).

Daechucyclopeptide-1 (*ca.* 2 mg) was derivatized to its acetate by usual method (pyridine-acetic anhydride). MS, *m/z* (rel. int.) 576 (M^+, 0.01), 533 (M^+-43, 0.1), 485 (M^+-91, 4.5), 429 (0.1), 332 (0.1), 287 (0.1), 290 (0.1), 261 (0.1), 245 (0.2), 244 (0.2), 219 (0.7), 193 (0.9), 181 (1.1), 151 (2.6), 148 (100), 96 (10.5), 68 (5.1).

The residue (52 mg) of Et_2O layer from fr.4 was purified by preparative TLC ($CHCl_3$:MeOH = 10:1) to yield daechualkaloid-E as crystaline powder (12 mg, Rf. 0.2). mp 154 °C, ^{1}H-NMR ($CDCl_3$, TMS) δ 2.24 (NH), 3.10-2.72 (6H,m, 4,5,7-CH_2 × 3), 3.67 (3H,s, 1-OCH_3), 3.88 (3H,s, 2-OCH_3), 6.64 (1H,s, 3-H), 7.29-7.22 (3H,m, 8-H, 9-H, 10-H), 8.36 (1H,m, 11-H). MS, *m/z* (rel. int.) 281 (M^+, 47.8), 280 (M^+-1, 80.6), 266 (M^+-15, 28.5), 252 (M^+-29, 7.5), 250 (M^+-31, 32.3), 237 (14.5), 221 (21.5). fr.4 (230 mg) was purified by preparative TLC ($CHCl_3$:MeOH = 10:1) to give Rf 0.2 compound (110 mg), which was acetylated by pyridine-acetic anhydride and purified by preparative TLC ($CHCl_3$:EtOAc:MeOH = 50:10:1) to yield daechualkaloid-E acetate (62 mg, needles from $CHCl_3$-Et_2O, yield 5.1×10^{-4}%). mp 204 °C (sublime). IR ν_{max}^{KBr} cm^{-1} 1630 (C = O). ^{1}H-NMR ($CDCl_3$, TMS) δ 2.18 (3H,s, N-$COCH_3$), 3.66 (3H,s, 1-OCH_3), 3.89 (3H,s, 2-OCH_3), 4.90 (1H, br, 6-H), 6.67 (1H,s, 3-H), 7.32-7.25 (3H,m, 8-H, 9-H, 10-H), 8.41 (1H, m, 11-H). MS, *m/z* (rel. int.) 323 (M^+, 6.5), 280 (1.9), 264 (M^+-59, 24.3), 252 (M^+-71, 25.5), 251 (M^+-72,100), 237 (12.3), 221 (6.3), 178 (9.7), 165 (19.4).

Isolation of zizyphusine

BuOH fr. (160 g) was subjected to silica gel column chromatography ($CHCl_3$:MeOH:H_2O = 15:10:2.5) to give Dragendorff reagent positive fraction (12 g). This alkaloid fr. was acetylated by pyridine-acetic anhydride. Reagents were removed under vacuum and the residue was suspended in water and washed with $CHCl_3$. The aqueous phase was concentrated to give 4g residue, which was purified by silica gel column chromatography $CHCl_3$:MeOH:H_2O = 15:10:2.5 to yield single compound (zizyphusine acetate, 498 mg). mp 214-5 °C, ^{1}H-NMR (DMSO-d_6, TMS) δ 2.17 (6H,s, 1,2 −$OCOCH_3$ × 2), 3.47, 2.96 (each 3H,s, N-dimethyl), 3.80 (3H,s, 11-OCH_3), 3.83 (3H,s, 10-OCH_3), 4.50 (1H,d, J = 12.1 Hz, 6a-H), 7.07 (1H,s, 3-H), 7.12 (1H,d, J = 7.3 Hz, 9-H), 7.35 (1H,d, J = 7.3 Hz, 8-H). MS, *m/z* (rel. int.) 426 (M^+, 2.6), 411 (5.8), 396 (1.6), 368 (1), 352 (1.6), 310 (1.6), 283 (1), 262 (1.6), 251 (1), 239 (1.6), 205 (2.6), 168 (3.1), 58 (96.3). Zizyphusine acetate (247 mg) was dissolved in MeOH (20 m*l*) and c-NH_4OH (20 m*l*), stood at room temperature for 3 hr. and the solvents were removed under vacuum. The residue was purified by silica gel column ($CHCl_3$:MeOH:H_2O = 15:10:2.5) to give zizyphusine (200 mg). mp 185-7 °C (decomp.), $[\alpha]_D$ + 312 (c = 0.4, H_2O). ^{1}H-NMR (DMSO-d_6, TMS) δ 3.28, 2.85 (each

3H,s, N-dimethyl), 3.68 (6H,s, 10,11-$OCH_3 \times 2$), 5.00 (br.s, OH × 2), 6.49 (1H,s, 3-H), 6.36 (1H,d, J = 7.8 Hz, 9-H), 6.58 (1H,d, J = 7.8 Hz, 8-H).

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