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Full Length Research Paper

Seven alkaloids and their antibacterial activity from *Hypecoum erectum* L.

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From the aerial part of *Hypecoum erectum* L., seven alkaloids, Protopine (1), Cryptopine (2), Allocryptopine (3), Hypecorinine (4), (-)-N-Methylcanadine (5), Oxohydrastinine (6) and N-Methylcorydaldine (7) were isolated. The structures of the seven compounds were established by ^{1}H-NMR, ^{13}C-NMR, DEPT and ESI-MS analyses. Compounds 2, 5 and 7 were obtained from this plant for the first time. The antibacterial activities were investigated against six bacteria (3 gram-positive and 3 gram-negative) by disc diffusion method followed by the minimal inhibitory concentration (MIC) which was determined using a two fold serial dilution assay for the compound which have inhibitory effect. *In vitro* antimicrobial activities assay indicated that some compounds showed promising activities. To the best of our knowledge, this is the first report on the antimicrobial properties of these alkaloids from this plant.

Key words: *Hypecoum erectum* L., alkaloids, antimicrobial activity.

INTRODUCTION

Hypecoum erectum L., an annual herb belonging to the Papaveraceae family, was widely distributed in dry and barren sandy land, roadsides and ditch land of Northeast China, Inner Mongolia, North China, etc (Li, 1987). Its root and whole plant are used as antipyretic and antitussive herbal medicine (He et al., 1984), as well as folk medicine employed in the treatment of acute pharyngitis and sore red eyes (Jiangsu Medical College, 1998). The pharmacognosy (Lei et al., 2003), pharmacology (Li and Han, 2006; Ke et al., 1987; Guo et al., 2006; Zhang et al., 2005), allelopathy (Wang et al., 2008), introduction and cultivation (Wang and Wang, 2004), methods of identification (Cai et al., 2007) of *H.erectum* L. were investigated and reported. The chemical constituents of *H. erectum* L. were mainly isoquinoline alkaloids, such as hypecorine and hypecorinine (Yakhontova et al., 1972), hyperectine (Perel'son et al., 1984), protopine, coptyine and allocryptopine (Yakhontova et al., 1984), isohyperectine (Yakhontova et al., 1993) and Oxohydrastine (Cai et al., 2007). Lei et al. (2003) found there were more types of chemical constituents of the aerial part of *H. erectum* L. than that of the underground. This paper described the isolation and the structural elucidation of seven (Protopine, Cryptopine, Allocryptopine, Hypecorinine, (-)-N-Methylcanadine, Oxohydrastinine and N-Methylcorydaldine) from the ethanol extracts of the aerial parts of *H. erectum* L. The seperation and purification were carried out through column chromatography and preparative TLC. The structures of the known compounds were confirmed by comparison of their spectroscopic data with those reported in the literature. The antimicrobial activity of the isolated compounds was investigated against *Bacillus cereus*, *Staphylococcus aureus* and *Bacillus subtilis* (gram-positive bacteria), and *Escherichia coli*, *Pseudomonas aeruginosa* and *Erwinia carotovora* (gram-negative bacteria) by disc diffusion method followed by the determination of minimal inhibitory concentration (MIC) through twofold serial dilution method for the bioactive compounds in the primary evaluation, to the best of our knowledge, this is the first report on the antimicrobial property of these alkaloids from this plant.

MATERIALS AND METHODS

Plant material

H. erectum L. was collected in Henan Province, People's Republic of China, in March, 2008, and identified by Prof. Chang ZY. A

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voucher specimen is deposited in Botanic Specimen Center of Northwest A&F University, Yangling, China.

Extraction and isolation

Air-dried aerial part of *H. erectum* L. (4.0 kg) were powdered and extracted by 70% aqueous ethanol at room temperature (1:10 W/V, 3 times, overnight each time), the pooled solvents were decolored with 3‰ active carbon, evaporated under reduced pressure to yield residue (555.0 gDW), which was resuspended in water and filtered. The filtrate was made alkaline (pH 9 to 10) with concentrated ammonia and repeatedly extracted with $CHCl_3$ to yield 67.7 g of $CHCl_3$-soluble total alkaloid extracts. (Monsef et al., 2004). The $CHCl_3$-soluble extract subsequently flowed through a column packed with D101 macroporous resin. The column adsorbing total alkaloids were eluted with water, 10, 30, 50, 70 and 95% ethanol water and finally EtOAc. After the solvent was removed at reduced pressure, 10% ethanol eluent gave 17.910 g alkaloid fraction A and 30% ethanol eluent provided 8.312 g alkaloid fraction B. The alkaloid fraction A was subjected to column chromatography over neutral alumina (200 to 300 mesh), using $CHCl_3$-MeOH (stepwise, 1:0, 100:1, 40:1, 20:1, 0:1) to afford fractions Fr.1~Fr.4. Fr.1 was repeatedly chromatographed on silical gel using petroleum-acetone (stepwise, 1:0, 20:1, 10:1, 8:1, 0:1) and recrystallized in the mixed solution of chloroform and methnol to give Protopine (1) (200 mg), Cryptopine (2) (30 mg), Allocryptopine (3) (200 mg), Hypecorinine (4) (130 mg). Fr.3 was repeatedly chromatographed on silical gel (200 to 300 mesh) using $CHCl_3$-MeOH (stepwise, 100:0, 50:1, 30:1, 20:1, 0:1) and recrystallized in the mixed solution of chloroform and methnol to give (-)-N-Methylcanadine (5) (300 mg).

The alkaloid fraction B was subjected to column chromatography over silical gel using $CHCl_3$-MeOH (stepwise, 100:1, 60:1, 40:1, 20:1,10:1 and 0:1) to afford fractions. Fr.5~Fr.8. Fr.5 was repeatedly chromatographed on silical gel using petroleum ether-acetone (stepwise, 100:1, 80:1, 50:1, 30:1 and 0:1) afford subfractions Fr.5.1~Fr.5.3, Fr.5.1 and Fr.5.2 were combined and repeatedly chromatographed on silical gel using petroleum ether-acetone and recrystallized in the mixed solution of chloroform and methnol to give Oxohydrastinine (6) (200 mg) and N-Methylcorydaldine (7) (300 mg), respectively.

Identification of compounds

Compound (1)

White crystal; mp: 205~206℃; ^{1}H-NMR(500MHz, d_6-DMSO, δppm): 6.95(1H, s, H-1), 6.80(1H, s, H-4), 2.42 (2H, m, H-5), 2.81 (2H, m, H-6), 1.82 (3H, s, N-CH_3), 3.51 (2H, m, H-8), 6.68 (1H, d, J=8 Hz, H-11), 6.72 (1H, d, J=8 Hz, H-12), 3.83 (2H, m, H-13), 5.96 (2H, s, O-CH_2-O), 5.99 (2H, s, O-CH_2-O). ^{13}C-NMR (125MHz, d_6-DMSO, δppm): 107.3 (C-1), 145.8 (C-2), 147.3 (C-3), 110.4 (C-4), 135.9 (C-4a), 30.4 (C-5), 57.4 (C-6), 41.1 (N-CH_3), 50.7 (C-8), 118.2 (C-8a), 145.3 (C-9), 145.2 (C-10), 106.3 (C-11), 125.1 (C-12), 129.6 (C-12a), 45.9 (C-13), 194.2 (C-14), 132.7 (C-14a), 101.1 (O-CH_2-O), 100.7 (O-CH_2-O). ESI-MS m/z: 354.08[M+H]$^+$. These data were similar to literature values (Guineudeau and Shamma, 1982; Wynne et al., 2004), and compound (1) was determined as Protopine.

Compound (2)

white crystal; mp: 220~221℃; ^{1}H-NMR (500MHz, d_6-DMSO, δppm): 1.79 (3H, s, N-CH_3), 2.49(2H, m, H-6), 2.89 (2H, m, H-5), 3.59 (2H,m, H-8), 3.89 (2H, br, H-13), 3.75 (6H, s, -OCH_3×2), 5.96 (2H, s, -OCH_2O-), 6.82 (1H, s, H-4), 6.71 (2H, m, H-11, 12), 6.93 (1H, s, H-1). ^{13}C-NMR (125MHz, d_6-DMSO, δppm): 106.3 (C-1), 146.4 (C-2), 148.8 (C-3), 111.7 (C-4), 134.3 (C-4a), 22.8 (C-5), 57.3 (C-6), 41.0 (N-CH_3), 50.4 (C-8), 130.0 (C-8a), 146.4 (C-9), 145.7 (C-10), 113.9 (C-11), 125.0 (C-12), 118.0 (C-12a), 45.8 (C-13), 194.8 (C-14), 131.2 (C-14a), 55.5 (2-OCH_3), 55.6 (3-OCH_3), 100.7 (-OCH_2O-). ESI-MS (+) m/z: 370.13 [M+H]$^+$. These data were similar to literature value (Guineudeau and Shamma, 1982), and compound (2) was determined as Cryptopine.

Compound (3)

Colorless crystal; mp: 162~163℃; ^{1}H-NMR (500MHz, d_6-DMSO, δppm): 6.92 (1H, s, H-1), 6.92 (1H, d, *J*=8Hz, H-11), 6.85 (1H, d, *J*=8Hz, H-12), 6.78 (1H, s, H-4), 5.98 2H, s, -OCH_2O-), 4.05 (2H, br. s, H-8), 3.76, 3.64 (both 3H, each s, 9-OCH_3, 10-OCH_3), 3.70 (2H, br. s, H-13), 3.02 (2H, br., H-5), 2.53 (2H, br., H-6), 1.76 (3H, s, N-CH_3). ^{13}C-NMR (125MHz, d_6-DMSO, δppm): δ108.0 (C-1), 145.2 (C-2), 147.2 (C-3), 110.3 (C-4), 132.8 (C-4a), 30.8 (C-5), 57.1 (C-6), 40.8 (N-CH_3), 50.1 (C-8), 128.8 (C-8a), 146.6 (C-9), 150.9 (C-10), 110.5 (C-11), 127.7 (C-12), 129.9 (C-12a), 45.8 (C-13), 193.1 (C-14), 135.8 (C-14a), 60.2 (-OCH_3), 55.4 (-OCH_3), 101.1 (-OCH_2O-); ESI-MS m/z: 370.12[M+H]$^+$. These data were similar to literature value (Jong-Ki et al., 2009), and Compound (3) was determined as Allocryptopine.

Compound (4)

Colorless crystal; mp: 196~198℃; ^{1}H-NMR (500MHz, d_6-DMSO, δppm): 6.41 (1H, s, H-1), 6.73 (1H, s, H-4), 3.17 (1H, m, H-5), 3.23 (1H, m, H-5), 2.88 (1H, m, H-6), 2.86 (1H, m, H-6), 4.71 (1H, d, *J*=15.3Hz, H-8), 5.05 (1H, d, *J*=15.3Hz, H-8), 7.07 (1H, d, *J*=8Hz, H-11), 7.60 (1H, d, *J*=8Hz, H-12), 2.19 (3H, s, N-CH_3), 5.95 (2H, m, -OCH_2O-), 6.21 (2H, m, -OCH_2O-). ^{13}C-NMR (125MHz, d_6-DMSO, δppm): 107.8 (C-1), 146.9 (C-2), 151.8 (C-3), 108.2 (C-4), 125.6 (C-4a), 23.3 (C-5), 44.8 (C-6), 56.6 (C-8), 124.3 (C-8a), 145.1 (C-9), 141.7 (C-10), 108.0 (C-11), 123.2 (C-12), 129.4 (C-12a), 191.4 (C-13), 90.7 (C-14), 122.3 (C-14a), 36.7 (N-CH_3), 100.8 (-OCH_2O-), 102.9 (-OCH_2O-); ESI-MS m/z: 367.88[M+H]$^+$. These data were similar to literature value (Hassan-Elrady, 1995), and Compound (4) was determined as Hypecorinine.

Compound (5)

Colorless crystal; mp: 255~256℃; ^{1}H-NMR (500MHz, d_6-DMSO, δppm): 7.15 (1H, d, *J*=7.5Hz, H-12), 7.11 (1H, d, *J*=7.5Hz, H-11), 7.09 (1H, s, H-1), 6.90 (1H, s, H-4), 6.06 (2H, m, -OCH_2O-), 4.85 (1H, d, *J*=15.6 Hz, H-8), 3.84 (3H, s, 9-OCH_3), 3.80 (3H, s, 10-OCH_3), 4.71 (1H, d, *J*=15.6 Hz, H-8), 3.27 (1H, m, H-14), 2.82 (3H, s, N-CH_3), 3.11 (1H, dd, *J*=18.1, 4.3 Hz,H-13a), 4.03 (2H, m, H-6), 3.77 (2H, m, H-5), 2.95 (1H, dd, *J*=18.1, 4.3 Hz, H-13b). ^{13}C-NMR (125MHz, d_6-DMSO, δppm): 105.9 (C-1), 147.0 (C-2), 147.3 (C-3), 108.4 (C-4), 124.4 (C-4a), 23.2 (C-5), 60.2 (C-6), 60.3 (C-8), 122.9 (C-8a), 144.8 (C-9), 150.7 (C-10), 113.4 (C-11), 124.4 (C-12), 124.2 (C-12a), 27.7 (C-13), 64.9 (C-14), 123.0 (C-14a), 38.7 (N-CH_3), 60.6 (2-OCH_3), 55.9 (3-OCH_3), 101.5 (-OCH_2O-); ESI-MS m/z: 354.20[M+H]$^+$. These data were similar to literature value (Jong-Ki et al., 2009), and Compound (5) was determined as (-)-N-Methylcanadine.

Compound (6)

Colorless crystal, mp: 90~93℃; ^{1}H-NMR (500MHz, CD_3OD, δppm): 3.53 (2H, t, *J*=7Hz, H-3), 2.89 (2H, t, *J*=7Hz, H-4), 6.69 (1H, s, H-5), 7.31 (1H, s, H-8), 5.97 (2H, s, -OCH_2O-), 3.08 (3H, s, N-Me). ^{13}C-NMR (125MHz, CD_3OD, δppm): 166.5 (C-l), 35.3 (N-Me), 49.3 (C-3), 28.6 (C-4), 123.9 (C-4a), 108.1 (C-5),152.2 (C-6), 148.3 (C-7), 108.2 (C-8), 135.8 (C-8a), 103.1 (-OCH_2O-); ESI-MS m/z:

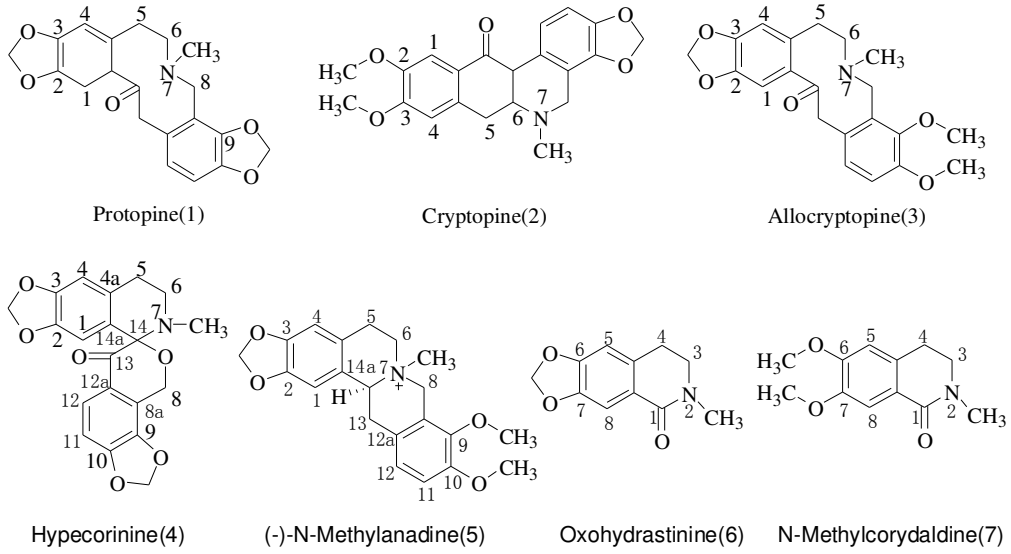

Figure 1. Structures of the alkaloids from *H.erectum* L.

227.94[M+Na]$^+$. These data were similar to literature values (Zhang et al., 1995; Fazal et al., 1983), and Compound (6) was determined as Oxohydrastinine.

Compound (7)

Colorless crystal; mp: 112~113℃; ^{1}H-NMR (500MHz, CD_3OD, δppm): 3.56 (2H, t, *J*=6.8Hz, H-3), 2.92 (2H, t, *J*=6.8Hz, H-4), 6.69 (1H, s, H-5), 7.45 (1H, s, H-8), 3.09 (3H, s, N-CH_3), 3.83, 3.85 (3H each, s, -OCH_3). ^{13}C-NMR (125MHz, CD_3OD, δppm): 166.9 (C-1), 49.4 (C-3), 28.1 (C-4), 134.1 (C-4a), 111.1 (C-5), 153.8 (C-6), 149.4 (C-7), 111.5 (C-8), 122.4 (C-8a), 56.4 (-OCH_3), 56.5 (-OCH_3); 35.3 (N-CH_3), ESI-MS m/z: 243.98[M+Na]$^+$. These data were similar to literature value (Tao et al., 2008), and Compound (7) was determined as N-Methylcorydaldine.

Tested microorganisms

The microorganisms, including three gram-positive bacteria (*B. cereus*, *S. aureus* and *B. subtilis*) and three gram-negative species (*E. coli*, *P. aeruginosa* and *E. carotovora*.) were kindly provided by the Institute of Pesticide of Northwest A&F University.

Antibacterial activity assay

The disc diffusion method (National Committee for Clinical Laboratory Standards, 2003) was applied to evaluate the antimicrobial activities of the foregoing chemical constituents. The media were inoculated with cell suspension (10^4 cfu/ml) and the discs were loaded with the tested alkaloids (Protopine, Cryptopine, Allocryptopine, Hypecorinine, (-)-N-Methylcanadine, Oxohydrastinine and N-Methylcorydaldine) dissolved in methanol (1 mg/mL). After evaporation of the loading solvent, each disc was placed at the center of a petri dish containing agar, previously inoculated with the specific microorganism. Incubation was continued for 18 h at a specific temperature (37±2℃). After these periods, the plates were examined and the inhibition zones were measured (diameter of the inhibition zone around each disc plus diameter of the disc). Streptomycin discs were used as positive controls. To rule out the activity of the solvent that was used as a vehicle, solvent-treated plates were maintained and served as a negative control and then replicated three times.

Determination of minimal inhibitory concentrations (MIC)

Six serial methanol dilutions from each compound (500, 250, 125, 62.5, 31.25 and 15.625 mg/ml) were prepared. Each concentration was mixed with nutrient agar for bacteria. All petri dishes including those containing the standard antibiotics (positive control) and the solvent vehicle only (negative control), were inoculated with the test organism by streaking the medium with a calibrated loop (0.05 ml). After incubation for 18 h (at 37±2℃), Petri dishes were examined for microbial growth and the minimum inhibitory concentrations (the minimum concentration of each treatment that inhibit microbial growth after the incubation period) were measured (Courvalin et al., 1985).

RESULTS AND DISCUSSION

Alkaloids from *H. erectum* L.

This paper describes the isolation and structure elucidation of the alkaloids of the ethanol extracts of the aerial parts of *H. erectum* L. The ethanol extracts of *H. erectum* L. were partial purified by partitioned extraction based on the polarity of organic solvents. The structures of the seven alkaloids were established by ^{1}H-NMR, ^{13}C-NMR, DEPT and ESI-MS analyses. The structures of the known alkaloids were confirmed by comparison of their spectroscopic data with those reported in the literature, three alkaloids, namely; Cryptopine, (-)-N-Methylcanadine and N-Methylcorydaldine were obtained from this plant for the first time. These seven alkaloids were shown in Figure 1.

Table 1. Antimicrobial activities of the alkaloids from the aerial part of *H. erectum* L.

Compounds	Inhibition zone (mm)						MIC (μg/ml)				
	Sa	Bc	Ec	Bs	Pa	Er	Sa	Bc	Ec	Bs	Pa
(1)	16	13	22	/	21	/	250	>500	125	/	125
(2)	10	9	/	/	/	/	>500	>500	/	/	/
(3)	18	12	20	10	19	/	250	>500	125	>500	125
(4)	15	13	19	/	21	/	250	>500	125	/	125
(5)	12	11	10	10	/	/	500	500	250	500	500
(6)	/	/	10	7	/	/	/	/	>500	>500	/
(7)	/	/	/	/	/	/	/	/	/	/	/
CK	18	26	20	21	/	/	1	1	0.0625	0.125	/
SC	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE

Bc-*Bacillus cereus*, Sa-*Staphylococcus aureus*, Bs-*Bacillus subtilis*, Ec-*Escherichia coli*, Pa-*Pseudomonas aeruginosa*, Er-*Erwinia carotovora*. CK-Streptomycin; SC-solvent control; ND-no effect. Values are the mean of three replicates and inhibition zone including the diameter of the bore (4mm).

Antibacterial activity of the alkaloids

The antibacterial activity of the alkaloids was evaluated against six categories of microorganisms, using disc diffusion and twofold serial dilution assay. The disc diameters of zone of inhibition, minimal inhibitory concentration (MIC) of the seven alkaloids against the microorganisms was tested (Table 1). As shown in Table 1, except for N-Methylcorydaldine (7), all the separated alkaloids possessed certain antimicrobial activity *in vitro*, in which alkaloids Protopine (1), Allocryptopine (3), Hypecorinine (4), showed more obvious antimicrobial properties than others. Though the antimicrobial properties of Protopine (1) and Allocryptopine (3) from other plant were reported (Pavel et al., 2010), to the best of our knowledge, this is the first report on the antimicrobial properties of these alkaloids from this kind of plant.

Conclusions

In the overall, the studies on *H. erectum* L. ethanol extracts have shown promising results, and further elucidation of the pharmacological role of the active constituents has become imperative. Further investigations are required in order to isolate more new compounds from the plant extracts, and their bioactivities still need to be explored on experimental basis.

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