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Phytochemical and biological activity studies of the Bhutanese medicinal plant corydalis crispa

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Abstract

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Keywords

biological, activity, studies, phytochemical, bhutanese, crispa, medicinal, plant, corydalis, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

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Phytochemical and Biological Activity Studies of the Bhutanese Medicinal Plant *Corydalis crispa*

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The chemical constituents and biological activities of *Corydalis crispa* (Fumariaceae) were investigated for the first time. The phytochemical study resulted in the isolation of nine known isoquinoline alkaloids: protopine (**1**), 13-oxoprotopine (**2**), 13-oxocryptopine (**3**), stylopine (**4**), coreximine (**5**), rheagenine (**6**), ochrobirine (**7**), sibiricine (**8**) and bicuculline (**9**), with complete NMR data for **2** and **3** provided here for the first time. Crude extracts exhibited significant anti-inflammatory ($p<0.01$) activity against TNF-α production in LPS activated THP-1 cells. The acetylcholinesterase inhibitory activity of compounds **2**, **4** and **7** and the antiplasmodial activity of compound **5** against *P. falciparum* strains TM4/8.2 and K1CB1 (multidrug resistant strain) are reported here for the first time. Stylopine (**4**) did not show antimalarial activity against the K1CB1 strain in contrast to a previous report. This study generated a scientific basis for the use of this plant in Bhutanese traditional medicine, either individually or in combination with other medicinal ingredients to treat a broad range of disorders. This study also identified compound **5** as potential new antimalarial lead compound.

Keywords: *Corydalis crispa*, isoquinoline alkaloid, Bhutanese traditional medicine, anti-inflammatory, antiplasmodial, anti-acetylcholinesterase.

The genus *Corydalis* (Fumariaceae) are a rich source of isoquinoline alkaloids which mediate chemical defence against a variety of micro-organisms and herbivores [1-2]. While few plant species of this genus were reported for cattle and sheep poisoning [1], many species are regarded as non-toxic and are being used in Asian folklore medicines for treating different types of ailments [3-4]. Owing to these interesting inherent properties, the *Corydalis* genus has been extensively studied. More than 58 *Corydalis* species have been studied phytochemically with over 190 isoquinoline alkaloids reported [5].

However, no phytochemical and biological activity studies on the Bhutanese medicinal plant, *C. crispa* have been reported. Locally, it is known as *ngo-ba-sha-ka* in Bhutan and contributes to the preparation of as many as 21 important multi-ingredient Essential Traditional Medicine Drugs (ETMDs) or polyherbal formulations [6]. As an individual plant, *C. crispa* is used in the Bhutanese traditional medicine (BTM) as a febrifuge and for treating infections in the blood, liver and bile which correlate to the signs and symptoms of parasitic and microbial infections, tumor and inflammatory conditions [7]. We previously reported the antiplasmodial, antimicrobial, anti-*Trypanosoma brucei rhodesiense* and cytotoxicity activities of the crude extracts of this plant [7]. In this study, since this plant is also ethnopharmacologically indicated for the management of various inflammatory conditions in Bhutan, we first assessed the anti-inflammatory potential of its crude extracts and then investigated the phytochemical components of this plant followed by bioassays on the major compounds with a view to derive potential new drug lead compounds and generate scientific evidence for its ethnopharmacological uses.

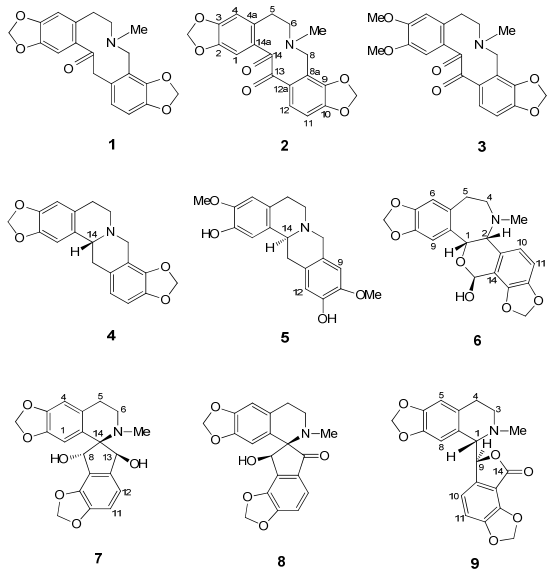

Figure 1: The structures of compounds **1-9.**

The phytochemical study of this plant resulted in the isolation of nine known isoquinoline alkaloids (Figure 1): protopine (**1**), 13-oxoprotopine (**2**), 13-oxocryptopine (**3**), stylopine (**4**), coreximine (**5**), rheagenine (**6**), ochrobirine (**7**), sibiricine (**8**) and bicuculline (**9**).

Compounds **1-4** belonged to the class of protopine alkaloids; compound **5** to the class of protoberberine alkaloids; compound **6** to the class of rhoeadine alkaloids; compounds **7** and **8** to the class of spirobenzylisoquinoline alkaloids; and compound **9** to the class of phthalide-isoquinoline alkaloids. The presence of more than one class of these alkaloids from a genus or even within a species has been reported [8]. Compound **6** has been reported previously from the genus of *Papaver*, *Meconopsis*, *Glaucum*, *Roemeria*, *Bocconia* and *Fumaria* [9-10]. However, this is the first rhoeadine-type

alkaloid being isolated from the genus *Corydalis* (Fumariaceae). Its presence in *C. crispa* is not that surprising since: a) rhoeadine alkaloids have been reported from its close relative *Fumaria parviflora* belonging to the same family of Fumariaceae; and b) rhoeadine alkaloids are the biogenetic derivatives of protopine [10]. Furthermore, direct evidence for the incorporation of labeled protopine into rhoeadine in *P. rhoeas* has been documented [11]. The literature on compounds **2** and **3,** lacked full NMR characterisation and we have presented here for the first time (Table 1).

13-Oxoprotopine (**2**) was previously characterized only by melting point, UV, IR and MS data [12]. Compound **2** was isolated here as a white amorphous solid. Its melting point, molecular formula and the major ion fragmentation pattern corresponded with the reported data for 13-oxoprotopine [12-13]. The ^{1}H-NMR spectrum of **2** showed the aromatic (CH) protons resonating at δ 7.43 (1H, s), 6.69 (1H, s), 6.83 (1H, d, J = 8 Hz), and 7.46 (1H, br s). The gHSQC showed these protons linked to the carbon signals resonating at δ 109.1 (C-1), 109.6 (C-4), 107.4 (C-11) and 125.9 (C-12), respectively.

Table 1: ^{13}C-NMR (125 MHz) and ^{1}H (500 MHz) data of Compounds **2** and **3**.

Position	**2** (in $CDCl_3$)		**3** (in $CDCl_3$)	
	δ C	δ H (mult., J (Hz)	δ C	δ H (mult., J (Hz)
1	109.1	7.43 s	111.8	7.48 s
2	145.1		147.0	
3	148.6		150.6	
4	109.6	6.69 s	112.4	6.70 s
4a	131.9*		130.7	
5	29.14	1.26 br s	29.2	1.64 br s
6	46.12*	2.65 br s	46.0*	2.64 br s
8	50.06	3.64 br s	50.1	3.67 br s
8a	120.0*		120.0	
9	146.0*		145.2	
10	147.0*		149.9	
11	107.4	6.83 d (8)	107.4	6.83 d (8)
12	125.9	7.46 br s	125.9	7.49 br s
12a	128.0*		128.1	
13, C=O	182.1*		182.0*	
14, C=O	191.7		191.9	
14a	131.5		131.0*	
N-CH_3	42.36	1.92 s	42.1	1.86 s
2-OCH_3	–		56.0	3.89 s
3-OCH_3	–		55.9	3.94 s
2,3-OCH_2O	101.3	5.99 s	–	–
9,10-OCH_2O	101.9	6.05 s	101.9	6.04 s

* Signal only observed in the gHMBC spectrum.

The two methylenedioxy protons were observed at δ 5.99 (2H, s) and 6.05 (2H, s) with its connected carbons resonating at δ 101.3 and 101.9, respectively. The methylene ($3\times CH_2$) protons attached to C-atoms of C-5, C-6 and C-8 resonated as very broad peaks in the region of δ 1.26-3.64. The N-methyl protons resonated at δ 1.92 (3H, s). The C-13 and C-14 carbonyl groups resonated at δ 182.1 and δ 191.7, respectively. The gHMBC defined the final structure **2**. To further confirm the structure **2**, it was synthesized by oxidation of compound **1** with mercuric acetate and the compound synthesized was found identical to 13-oxoprotopine (**2**) by TLC, MS and ^{1}H-NMR analysis.

13-Oxocryptopine (**3**) was reported only from the family papaveraceae (*P. somniferum*) [9, 12] and this is the first report from the genus *Corydalis* (Fumariaceae). It was previously identified based on its melting point, MS and ^{1}H-NMR data [12,14] and its ^{13}C-NMR data has been lacking. Here, this compound **3** was obtained as an optically inactive pale green solid. The molecular formula, ion fragmentation pattern and the ^{1}H-NMR data were consistent with those reported for 13-oxocryptopine (**3**) [12, 14]. The APT spectra indicated the presence of three methyl groups, four methylene, four methine and ten quaternary carbons. From the *g*HSQC spectrum the four aromatic protons (CH) correlated to C-1, C-4, C-11 and C-12 carbons whose signals resonated at δ 111.8, 112.4, 107.4, and 125.9, respectively. A signal at δ 42.1 was assigned to the N-methyl carbon and the signals at δ 55.9 and 56.0 were assigned to the two methoxy carbons. The carbon of the methylenedioxy group resonated at δ 101.9. The long range *g*HMBC correlation established the structure **3**.

The other known compounds were identified through MS library matching techniques (NIST08s) and then confirmed through comparison of their melting point, optical rotation, mass and NMR spectra with those reported in the relevant literature. Compound **1** (950 mg) was identified as protopine [15-17], compound **4** (5 mg) as stylopine [15, 18-20], compound **5** (1 mg) as coreximine [21], compound **6** (1 mg) as rheagenine [10], compound **7** (60.6 mg) as ochrobirine [22-25], compound **8** (0.8 mg) as sibiricine [8, 23] and compound **9** as bicuculline [26-27].

In this study, prior to the investigation of its phytochemical contents and the biological activities of the major alkaloids, we first assessed the anti-inflammatory potential of its crude extracts against pro-inflammatory cytokine, tumor necrosis factor-α (TNF-α) in LPS activated THP-1 cells. The uncontrolled production of this pro-inflammatory cytokine by the immune system in response to infection, stimulates the over production of reactive oxygen species (ROS) which have been implicated in the pathogenesis of many forms of disease [28]. Thus, the pro-inflammatory cytokine (TNF-α) in LPS activated THP-1 cells are commonly used for evaluating the anti-inflammatory effects of various materials.

The anti-inflammatory assay (Table 2) demonstrated the level of TNF-α secretion of THP-1 monocytic cells and the percentage of TNF-α inhibition when incubated with crude extracts and pure alkaloids of the plant in the presence of LPS. The cytotoxic effects of the crude extracts and pure alkaloids on THP-1 cells were determined first on test samples at different concentrations. This cell viability test compositely indicated that, at 50 µg/mL of crude extracts and 3.9 µg/mL of pure alkaloids, there were no significant cell reduction. The percentage of viable cells in each sample was >90%. The percentage cell viability of RPMI was 97%, DMSO was 98% and dexamethasone was 97%. This suggested that the inhibition of TNF-α production by the test samples were not associated with their cytotoxic effects to the THP-1 cells. The THP-1 cells in RPMI and THP-1 cells incubated with the crude plant extracts or pure alkaloids alone (without LPS) did not activate TNF-α production at the above mentioned non-cytotoxic concentrations. In the presence of each of the plant extracts and individual alkaloids, the TNF-α production was either suppressed or stimulated in varying degrees as compared to the secretion levels of TNF-α production of RPMI culture media control and the DMSO solvent control (Table 2).

Among the four different solvent extracts, the hexane and dichloromethane extract showed significant anti-inflammatory activity with the suppression of TNF-α production to the values of 142 pg/ml (77% inhibition) and 71 pg/ml (89% inhibition), respectively (Figure 2). The suppression of TNF-α production of these two crude extracts were much higher than the DMSO control and the positive control drug dexamethasone (Table 2). The chloroform extract also showed suppression of TNF-α production to the value of 284 pg/mL (54% inhibition). A Student's t-test with one-tailed distribution showed that the results from the hexane and dichloromethane extracts (p<0.01) and the chloroform extract (p<0.05) were statistically significant (Figure 2). These significant

activities suggested that these crude extracts possess compounds with promising anti-inflammatory properties.

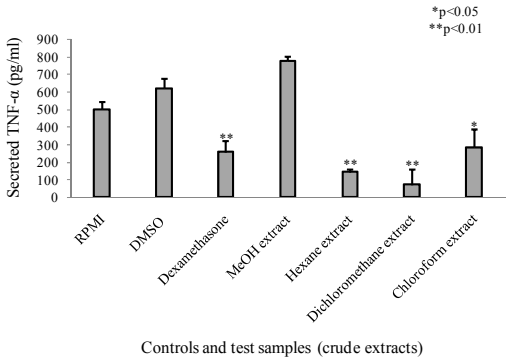

Figure 2: Anti-inflammatory effects of crude extracts on TNF-α production in LPS-activated THP-1 monocytic cells. RPMI as cell culture media control; DMSO as solvent control; Dexamethasone as positive control drug; asterisks denote statistical significance in relative to solvent control; *($p<0.05$); **($p<0.01$); error bars indicate standard deviations.

Guided by the potent biological activities of its crude extracts, out of nine isolated compounds, we investigated six major compounds **1**, **2**, **4**, **5**, **7** and **9** for their anti-inflammatory, antiplasmodial, antimicrobial, anti-acetylcholinesterase, anti-*Trypanosoma brucei rhodesiense* and cytotoxicity activities (Table 2 and Table 3). Compound **1** exhibited significant anti-inflammatory, anti-acetylcholinesterase and the antiplasmodial activities against *Plasmodium falciparum* strains: TM4/8.2 (a wild type chloroquine and antifolate sensitive strain) and K1CB1 (multidrug resistant strain) (Table 2). It also showed broad range of mild level antimicrobial activity against different bacterial strains as compared to other test compounds (Table 3). The antiplasmodial, antimicrobial, and anti-inflammatory activities of this compound were found in line with the previously reported data [15, 29-32]. Compounds **2** and **4** displayed stimulated effects on TNF-α production and moderately inhibited acetylcholinesterase. Kaya et al. [33] reported that compound **4** (stylopine) had significant antimalarial activity against the drug resistant *P. falciparum* K1 strain with IC_{50} values of 0.23 µg/mL. However, we found that compound **4** was inactive even at the highest concentration of 4–4.6 µg/mL. Indeed, previous studies indicated that compound **4** was inactive against *P. berghei* and *P. falciparum* [15, 34-35]. Compound **5** showed significant antiplasmodial activity against TM4/8.2 and K1CB1 strains with IC_{50} values of 5.56 ± 0.63 µg/mL and 6.87 ± 1.09 µg/mL, respectively. Thus, it has the potential to be a new candidate for antimalarial drug lead. This compound also moderately inhibited acetylcholinesterase (Table 2) and inhibited *Helicobacter pylori* and *Bacillus subtilis* with the MIZ of 6 and 5 mm, respectively (Table 3). This MIZ values were significant when compared to that of the positive control, amoxicillin which inhibited the same strain with a MIZ value of 7 and 10 mm. However, they both showed only weak MIC values of 125 µg/mL. In fact, all the alkaloids tested here showed weak antibacterial activity against *H. pylori* with MIC values of 125 µg/mL. Compound **7** showed weak anti-inflammatory and moderate anti-acetylcholinesterase activities.

The anti-inflammatory, antimicrobial, antiplasmodial, anti-*Trypanosoma brucei rhodesiense* and the cytotoxicity activities of the crude extracts were very interesting for the fact that this plant is being used in the Bhutanese traditional medicine (BTM) as a febrifuge and for treating infections in the blood, liver and bile which correlate to the signs and symptoms of parasitic and microbial infections, tumor and inflammatory conditions [7].

Table 2: Bioactivities of the crude extracts and the pure compounds **1**, **2**, **4**, **5**, **7**, and **9** isolated from *C. crispa.*

	Anti-inflammatory		AChE[a]	Antiplasmodial (*P. falciparum*)		Anti-Tbr[b]	Cytotoxicity	
Samples	TNF-α (pg/mL)	Activity (%)	MIR (nmol)	TM4/8.2 IC_{50} (µg/mL)	K1CB1 IC_{50} (µg/mL)	IC_{50} (µg/mL)	Vero cells IC_{50} (µg/mL)	KB cells IC_{50} (µg/mL)
MeOH extract	778 ±3	+ (25)	Nt	11.53 ±4.83	13.24 ±0.59	>10	>25	>25
Hexane extract	142 ±17	− (77)	Nt	14.57 ±1.76	15.82 ±3.94	>10	>25	>25
CH_2Cl_2 extract	71 ±17	− (89)	Nt	12.09 ±0.58	11.27 ±3.14	>25	>25	>25
$CHCl_3$ extract	284 ±10	− (54)	Nt	2.11 ±0.55	1.89 ±0.20	4.63 ±0.53	>25	12.50
1	467 ±4	− (25)	0.14	1.45±0.53	1.38±0.31	>3.5	>3.5	>3.5
2	862 ±14	+ (39)	1.31	>4.6	>4.6	>3.7	>3.7	>3.7
4	679 ±28	+ (10)	0.15	>4.0	>4.0.	>3.2	>3.2	>3.2
5	632 ±4	+ (1)	0.53	5.56±0.63	6.87±1.09	>8.2	>8.2	>8.2
7	606 ±30	− (2)	0.14	>9.2	>9.2	>9.2	>9.2	>9.2
9	791 ±11	+ (28)	>2.72	>9.2	>9.2	>9.2	>9.2	>9.2
RPMI	502±43							
DMSO	620±54							
Dexamethasone[c]	258 ±65							
Galanthamine[d]			0.003					
Chloroquine[e]				0.0096	0.089± 0.01			
Cycloguanil[e]				0.0095	0.81 ± 0.19			
Pyrimethamine[e]				0.0200	7.7 ± 2.14			
PB113[f]						0.064		
Ellipticine[g]							0.093	
Doxorubicin[g]								0.56

[a] Acetylcholinesterase inhibitory activity. [b] Anti-*Trypanosoma brucei rhodesiense* activity. Nt: Not tested; +: activated; −: inhibited.
[c] Reference drug for anti-inflammatory activity.[d] Reference drug for acetylcholinesterase activity. [e] Reference drugs for antiplasmodial activity.
[f] Positive control for anti-*Trypanosoma brucei rhodesiense* activity. [g] Positive controls for cytotoxicity activity.

Table 3: Antimicrobial activities of the crude extracts and the pure compounds of *C. crispa* (Minimum Inhibition Zone (MIZ) in mm, well diameter = 4 mm; Minimum Inhibitory Concentration (MIC) in µg/mL).

	Bacterial and fungal strains															
Samples	*V. cholerae*		*S. aureus*		MRSA		*E. coli*		*B. subtilis*		*S. epidermidis*		*H. pylori*		*C. albicans*	
	MIZ	MIC	MIZ	MIC	MIZ	MIC	MIZ	MIC	MIZ	MIC	MIZ	MIC	MIZ	MIC	MIZ	MIC

MeOH extract	5.5	1250	–	–	–	–	6	1250	–	–	5	1250	5.3	500	–	–
Hexane extract	6	2500	6	2500	6	2500	5.5	1250	–	–	6	2500	6.5	500	–	–
CH_2Cl_2 extract	5	1250	6	2500	5.5	1250	6	1250	5.8	625	6	1250	6.3	500	–	–
$CHCl_3$ extract	7	625	7	1250	7	1250	7	1250	7	625	7	625	6.5	500	5.5	313
1	7.5	250	8	250	5.5	250	7	250	–	–	–	–	5.3	125	Nt	Nt
2	Nt	Nt	4.5	500	4.5	500	6	250	5	250	Nt	Nt	5	125	Nt	Nt
4	Nt	Nt	5.5	250	5	500	6	250	5	250	Nt	Nt	5	125	Nt	Nt
5	Nt	Nt	5.5	250	4.5	250	6	250	5	125	Nt	Nt	6	125	Nt	Nt
7	Nt	Nt	4.5	500	4.5	250	5	250	5	250	Nt	Nt	5	125	Nt	Nt
9	Nt	Nt	6	500	4.5	250	5	250	5	250	Nt	Nt	5	125	Nt	Nt
Amoxicillin[a]	6	7.8	31	< 1			9	8	10	63	20	16	7	16		
Vancomycin[a]					15	4										
Amphotericin B[b]															18	<1

[a] Standard drug as a positive control for antibacterial testing.[b] Standard drug as a positive control for antifungal testing.
Nt: Not tested, −: Not active.

Thus, our study verified an ethnopharmacological uses of *C. crispa* and strongly suggested that this plant can be a source of broad spectrum, biologically active natural products. Unlike the crude chloroform extract of this plant, none of the six pure alkaloids tested here exhibited anti-*Trypanosoma brucei rhodesiense* and cytotoxicity activities. The anti-acetylcholinesterase activity of compounds **2**, **4** and **7**, and the antiplasmodial activity of compound **5** are reported here for the first time. Since compounds **1** and **5** exhibited significant antiplasmodial, moderate acetylcholinesterase inhibition and mild level antimicrobial activities, it may be deduced that these two alkaloids (in alone or synergistically) may be responsible for the major activity of this plant. It is also possible that other minor alkaloids which were isolated here (but not tested) and the non-alkaloid components which we could not readily isolate may be responsible for the broad range of interesting biological activities of the crude extract of a plant.

In summary, our study found the following: a) protopine (**1**) (950 mg/2 kg dry weight) was the major alkaloid of *C. crispa*, b) complete NMR data for compounds **2** and **3** were lacking which we furnished here for the first time, c) compound **5** showed significant *in vitro* antiplasmodial activity without cytotoxicity and was identified as a potential scaffold that could generate more leads with enhanced antiplasmodial activity, and d) the *in vitro* biological activities were in alignment with the ethnopharmacological uses of this plant and thus substantiate its use in BTM, either individually or in combination with other medicinal ingredients, to treat a broad range of disorders.

Experimental

General: A rotary evaporator was used for solvent evaporation under reduced pressure at 35 °C-50 °C. Flash column chromatography packed with Merck Kieselgel 60 PF_{254} was used for separation and purification of alkaloids. Aluminium-backed silica plates (0.2 mm silica thickness, Merck) were used for separating isolates of smaller quantities. UV light (short wavelength of 254 nm, long wavelength of 366 nm) and Dragendorff's reagent were used for visualization and detection of the separated alkaloid-rich bands on TLC plates. Micromass Waters Platform LCZ (single quadrupole, MeOH as solvent) was used for obtaining the LR-ESI-MS. Shimadzu GCMS-QP-5050 was used for recording the LR-EI-MS by the direct insertion technique (at 70 eV). Micromass Waters Q-ToF Ultima (quadrupole time-of-flight) mass spectrometer was used for acquiring HR-ESI-MS. A 500 MHz Varian Unity Inova, 500 MHz Varian Premium Shield (VNMRS PS 54), and 300 MHz Varian Mercury spectrometer were used for obtaining the NMR spectra (^{1}H-NMR, gCOSY, ^{13}C-NMR, APT, gHMBC, gHSQC, and gNOESY) using deuterated solvents (CD_3OD or $CDCl_3$) depending upon the solubility of compounds**.** IR spectra were obtained using a Smart Omni-Sampler Avator ESP Nicolet spectrometer. Reichert hot-stage apparatus was used for determining the melting point of the compounds. A JASCO 2000 Series polarimeter was used for measuring the optical rotations of the compounds using a sodium lamp and averages of ten optical readings were taken to obtain the observed rotation value.

*Plant material***:** *C. crispa* Prain, is a perennial yellow flowering herb that grows to about 10-23 cm tall arising from stout woody rootstock [36-37]. The whole part of wild *C. crispa* was collected from Thuphu (Altitude: 3962 m; Latitude: 27° 51' 15.4"; Longitude: 89° 27' 12.8"; global positioning system (GPS) point number: 187; Site number: P187; Slope: 40°; Aspect: South-East), under Lingshi block in Bhutan in August 2009. The collected plant material was air-dried and a herbarium specimen with voucher number 78 was deposited at the herbarium of the PRU, Thimphu, Bhutan.

Extraction and isolation: The air-dried plant material (2 kg) was chopped into small pieces and was repeatedly extracted with methanol (AR/HPLC grade, 5 × 3 L over 48 h). The extract was filtered and then concentrated using a rotary evaporator to afford the crude methanol extract (58.22 g). The MeOH extract (10 g) was acidified with 5% HCl and then subjected to sequential fractionation and extraction using hexane (5 × 60 mL) and CH_2Cl_2 (5 × 60 mL). Two fractionated extracts were concentrated resulting into hexane extract (1.34 g) and the CH_2Cl_2 extract (2.90 g). The remaining acidified aqueous solution was basified (pH 9-11) with NH_4OH solution, then extracted with $CHCl_3$ (5 × 60 mL). The $CHCl_3$ extract was washed (H_2O), dried (Na_2SO_4) and the solvent was evaporated under reduced pressure to yield a crude alkaloid-rich $CHCl_3$ extract (80 mg). Dragendorff's reagent was prepared [38] for staining the TLC plate loaded with alkaloid. A small portion of each of these crude extracts (40 mg each) was used for biological activity studies. Each extract obtained above was then subjected to fractional crystallization (mostly methanol/chloroform) followed by repeated separation and purification processes using column chromatography on silica gel (mobile phase: gradient eluant of increasing solvent polarities mainly using MeOH (1-30%) and $CHCl_3$) until pure alkaloids were obtained. Fractions of smaller quantities and those compounds with closer R_f values were separated repeatedly using Preparative Thin Layer Chromatography. Finally, nine known isoquinoline alkaloids **1**–**9** (Figure 1) were isolated.

13-Oxoprotopine (2)
17.6 mg (0.00088 % dry weight), white amorphous solid.
MP: 226-228 °C.
$[\alpha]_D$: inactive.
^{1}H-NMR and ^{13}C-NMR: see Table 1.
LR-ESI-MS (*m/z*): 368 [$M+H^+$].
LR-EI-MS (*m/z*): 367 [$M^{+\bullet}$], 295, 279, 257, 237, 209, 190, 162 (100%), 149, and 134.
HR-ESI-MS (*m/z*): 368.1159 [$M+H^+$] (354.1129 calc. for $C_{20}H_{18}NO_6^+$).

13-Oxocryptopine (3)
4.5 mg (0.000225% dry weight), pale green solid.
MP: 180-183 °C.
$[\alpha]_D$: inactive.
IR (KBr): vmax 792, 939, 1042, 1239, 1365, 1454, 1484, 1656 cm^{-1}.
^{1}H-NMR and ^{13}C-NMR see Table 1.
LR-ESI-MS (*m/z*): 384 $[M+H^+]$.
LR-EI-MS (*m/z*): 383 $[M^{+\bullet}]$, 340, 312, 297, 277, 253, 220, 204, 190, 162 (100%), 149 and 134.
HR-ESI-MS (*m/z*): 384.1461 $[M+H^+]$ (384.1442 calc. for $C_{21}H_{22}NO_6^+$).

Anti-inflammatory assay: The methanol, hexane, dichloromethane and chloroform extracts of *C. crispa* were prepared using the method described by Wangchuk et al., [7]. Cell culture and *in vitro* bioassays were performed as previously described [39]. Cells were incubated with crude plant extracts and pure compounds of *C. crispa* alone or in combination with purified LPS (100 ng/mL) from *Escherichia coli* serotype O127 : B8 (Sigma, USA) for 3.5 hr. Supernatants were collected from individual wells by centrifugation and assayed for TNF. The cell viability or the cytotoxic effects of crude extracts and pure compounds in THP-1 cells were determined by the Trypan-blue (Gibco-Invitrogen, USA) stain exclusion assay. Cells were incubated with varying concentration of crude extracts as 0, 25, 50, 100 and 200 µg/mL and that of pure compounds as 0, 1.95, 3.9, 7.8, 15.6 and 31.25 µg/mL. The concentration that showed the highest cell viability (without significant cell reduction) was chosen as the final concentration for crude extracts (50 µg/mL) and pure compounds (3.9 µg/mL). Cell suspension was mixed with Trypan Blue which stained dead cells. The viable and dead cells were counted with a hemacytometer under an inverted microscope. Cell viability was determined from the ratio of viable cells over total cells. Results were expressed as means with standard deviations (± SD). The percentage of cell viability was calculated by the formula:

% Cell viability = 100 × (1−(dead cells/total cells).

TNF-α production in THP-1 cell culture supernatants were measured using cytokine-specific sandwich quantitative enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions (R & D Systems, USA). The 96-well microtiter plates (Corning, USA) were coated overnight with mouse anti-human TNF-α antibody as capture antibodies. The plates were washed with PBST (0.05% Tween 20) to remove excess capture antibody. To reduce non-specific binding, wells were blocked for 2 h with bovine serum album (BSA) (1% (w/v) of BSA; Sigma, USA)) and washed with PBST. Recombinant human TNF-α was used as standard at the concentration of 15.6, 31.5, 62.5, 125, 250, 500, and 1000 pg/mL. The standard or samples were added to appropriate wells and plates were incubated overnight. After washing, biotinylated goat anti-human TNF-α antibodies were added as detection antibodies and the mixture was incubated for 2 h. The plates were washed with PBST and incubated for 20 min with streptavidin-horseradish peroxidase conjugate. The tetramethylbenzidine (TMB) substrate was added to the plates as a color indicator and was incubated for 20 min. H_2SO_4 was added to stop the reaction. Absorbance was measured at 450 nm using a BioTek® Synergy™ HT (Multi-Detection Microplate Reader, USA). Cytokine concentration in each well was quantified from a standard curve and expressed as pg/mL of culture medium.

The commercial anti-inflammatory drug, dexamethasone (10 µg/mL), (Atlantic LabsComp. Ltd., Thailand) was used as a positive control. The experiments were performed three times in triplicate (3×3). Results were expressed as means with standard deviations (± SD). The percentage of TNF-α inhibition was calculated by the formula:

% TNF-α inhibition = 100 × ((observed/baseline) − 1).

Where observed = secreted TNF-α of experiment (pg/mL) and baseline = secreted TNF-α of DMSO (pg/mL).

Statistical significance or differences were evaluated by GraphPad Prism version 5.01 using Student's *t*-test with one-tailed distribution. A p-value ≤ 0.05 was considered statistically significant.

Acetylcolinesterase inhibitory assay method: A rapid TLC bioautographic method described by Marston et al. [40] was used for acetylcholinesterase inhibitory testing. Galanthamine, which is a frontline AChE inhibitor used to treat Alzheimer's disease, was used here as a reference compound. The tests were performed in triplicate.

Other bioassay methods: The antimicrobial, antiplasmodial, anti-*Trypanosoma brucei rhodesiense* and cytotoxicity assays were carried out using the methods, test strains and references/standard drugs as described by Wangchuk et al. [7].

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