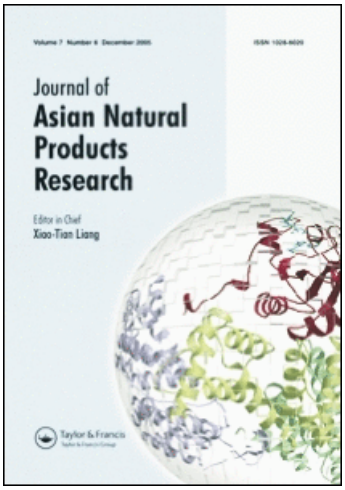

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ORIGINAL ARTICLE

Anti-hyperglycemic effect of 11-hydroxypalmatine, a palmatine derivative from *Stephania glabra* tubers

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A palmatine derivative, named 11-hydroxypalmatine (**4**), has been isolated from the tubers of *Stephania glabra*, together with three known alkaloids, palmatine (**1**), dehydrocorydalmine (**2**), and stepharanine (**3**). The structures of the compounds were elucidated by means of spectroscopic analysis including 2D NMR experiments. The hypoglycemic activity of **4** was evaluated against alloxan-induced diabetic mice. The test compound was administered at doses of 25, 50, and 100 mg/kg, p.o., 36 h after alloxan injection (60 mg/kg, i.v.). The alloxan-induced diabetic mice showed significant reduction in blood glucose after treatment with the test compound by 52% as compared to the positive control glibenclamide (54%) and the diabetic control (27%).

Keywords: *Stephania glabra*; Menispermaceae; hydroxypalmatine; alloxan; protoberberine alkaloids; anti-hyperglycemic activity

1. Introduction

Diabetes mellitus is one of the chronic, worldwide heterogeneous, and life-threatening diseases. Its prevalence will be 5.4% by the year 2025, with the global diabetic population reaching to 300 million. Among all the WHO regions, Southeast Asian region is the highest affected with maximum global burden of the disease and, by the year 2025, there will be nearly 80 million diabetics in the region [1,2]. Given the pandemic spread of type II diabetes, the identification of new therapeutic avenues in the treatment of all pathological aspects of this disorder remains a major challenge for current biomedical research.

A number of plants present in nature possess marked hypoglycemic activity. The extracts obtained from these plants have a significant activity to treat the disease but sometime showed toxicity. However, the improvement in potency and reduction of side effects of these extracts can be made either by the purification of the crude extract or by the isolation of the individual constituent responsible for such activities.

Stephania glabra (Menispermaceae) is a large, climbing shrub, indigenous to the

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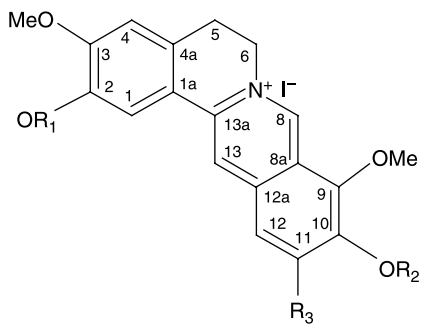
lower Himalaya of India. The tubers of the plant are used in India for the treatment of a variety of disorders, including asthma, tuberculosis, dysentery, and fever, and it is also used as a psycomedicine by natives [3–5]. According to the preliminary laboratory screening, the ethanolic extract of the plant tubers was found to be an antidiabetic. Therefore, the isolated constituents were evaluated for their anti-hyperglycemic activity. The tubers of the plant contain a variety of alkaloids such as aporphines, bisbenzylisoquinolines, berberines, and protoberberines [6–9]. The ethanolic extract of the tuber furnished four protoberberine alkaloids, in which palmatine (**1**), dehydrocorydalmine (**2**), and stepharanine (**3**) were previously isolated from this source [10] but 11-hydroxypalmatine (**4**) is perhaps isolated for the first time. Recently, we have reported two novel alkaloids from this source [11,12] and now report the isolation, characterization, and anti-hyperglycemic activity of 11-hydroxypalmatine.



	R_1	R_2	R_3
1	Me	Me	H
2	Me	H	H
3	H	H	H
4	Me	Me	OH

Figure 1. Chemical structure of isolated compounds.

2. Results and discussion

Compound **4** was isolated as yellow needles, mp 228–230°C, and its molecular formula, $C_{21}H_{22}NO_5I$, was determined from the molecular ion peak at *m/z* 495.3 in EI-MS. It gave positive reactions with Mayer's, Dragendorff's, and Wagner's reagents, indicating its alkaloidal nature. The reaction with silver nitrate, affording a yellow precipitate (insoluble in NH_4OH), was suggestive of an iodide salt. The ^1H NMR spectrum gave the evidence of four methoxy (3H singlets at δ 4.12, OMe-9; 4.03, OMe-3; 3.94, OMe-10; and 3.88, OMe-2), a hydroxy (1H singlet at δ 9.81, OH-11), and five aromatic protons (1H singlets at δ 8.79, H-8; 7.95, H-12; 7.78, H-13; 7.57, H-4; and 6.88, H-1). ^{13}C NMR and DEPT spectra showed 21 signals including four methoxy singlets at δ 55.4 (OMe-2), 55.6 (OMe-3), 56.0 (OMe-9), and 56.5 (OMe-10). The reaction with zinc dust yielded palmatine (compared with an authentic sample). Hence, the positions of all methoxy groups were similar to that of palmatine (Figure 1); this fact was further corroborated by HMBC correlations (Figure 2). The position of the hydroxy group was confirmed by the HMBC spectrum, which showed the correlation of hydroxy proton (δ 9.81) to C-10 (δ 143.7) and C-12 (δ 110.6). The LC-EI-MS (positive mode) exhibited a molecular ion at 495, which loses iodide to furnish an ion at 368 $[M^+ - I^-]$. The most abundant ion at 352 $[C_{20}H_{18}NO_5]^+$ is presumably

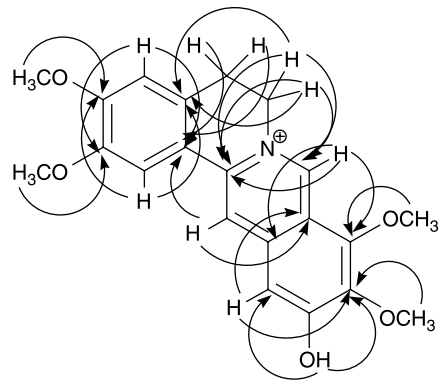


Figure 2. Important HMBC correlations of **4**.

derived by the loss of a methane molecule from 388. The ions at 192 $[C_{11}H_{14}NO_4]^+$ and 149 $[C_8H_5O_3]^+$ are probably due to the rupture of the ring C (Figure 3).

The results of the hypoglycemic effect in all groups of experimental animals are shown in Table 2. The hypoglycemic effects of **4** with doses of 25, 50, and 100 mg/kg, p.o., just prior to the third hour, were found to be significant in comparison with diabetic control animals. From third to sixth hour, a rapid increase in the blood glucose level was observed for all doses. A gradual decrease in the blood glucose level was observed from 6th to 24th hour. The results showed that the drug is the most effective at high concentration, i.e. 100 mg/kg. At 24th hour, the blood glucose level was found to be less than that of the diabetic control group and, in general, near to the positive control group (i.e. 100 mg/kg, p.o.). In the diabetic control group, the blood glucose level remained high during the experimental period. The mice treated with **4** showed significant hypoglycemic effects after the sixth hour, suggesting that it has long duration of hypoglycemic action. The overall performances of all groups in lowering the blood glucose level were 21% (normal control), 27% (diabetic control), 52% (diabetic+HP-100), 36% (diabetic+HP-50), 31% (diabetic+HP-25), and 54% (positive control). Compound **4** was found to be safe for further biological studies because of no lethality or other behavioral changes were observed up to 500 mg/kg per oral from LD_{50} experiments.

Alloxan produced a significant increase in the blood glucose level by damaging pancreatic β-cells, resulting in a decrease in endogenous insulin secretion, which decreases the utilization of glucose by the tissues and thus called an effective diabetes-induction agent [13]. Alloxan injection consistently produces symptoms of diabetes mellitus including hyperglycemia, decreased insulin levels, and polyuria. The blood glucose lowering effect of the 11-hydroxypalmatine was administered in alloxan-induced diabetic mice. In the diabetic control group, the blood glucose level was decreased by 27%, whereas the test compound decreased the blood glucose level by 52% (100 mg/kg), which was most significant than the diabetic control group. The positive control reduced the blood glucose level by 54%, which is almost the same as that of 100 mg/kg, p.o. The biphasic effect of **4** has been observed with maximum hypoglycemic effect at 24th hour, whereas the level was increased constantly 24 h onwards. On the basis of the above findings and discussion, it may be concluded that **4** is promising for developing standardized natural medicine for diabetes mellitus.

3. Experimental

3.1 General experimental procedures

The UV spectrum was recorded on a Perkin-Elmer, Lambda-25 spectrometer (methanol as the solvent), IR on Perkin-Elmer, and Spectrum RX I FT-IR spectrometer (KBr disks). NMR spectra were obtained on a JEOL NMR spectrometer (300 MHz for ^1H and 125 MHz for ^{13}C, DMSO-d_6 as the solvent, TMS as the internal standard). MS was recorded on a Finnigan MAT spectrometer (Xcalibur ver-2 software; Finnigan MAT, San Jose, CA, USA). TLC was carried out on silica gel (Merck 10–40 μ) precoated plates and visualized by spraying with Dragendorff's reagent. Alloxan was obtained from Central Drug House (P) Ltd (New Delhi, India) and Glibenclamide from Ind-Swift Ltd (Parwanoo, India).

3.2 Plant material

Fresh tubers (10 kg) of *S. glabra* were collected from Chaka, nearby Chandravadani temple (Tehri Garhwal) during October 2006 and identified at Taxonomy Laboratory, Department of Botany, H.N.B. Garhwal University Srinagar. A voucher

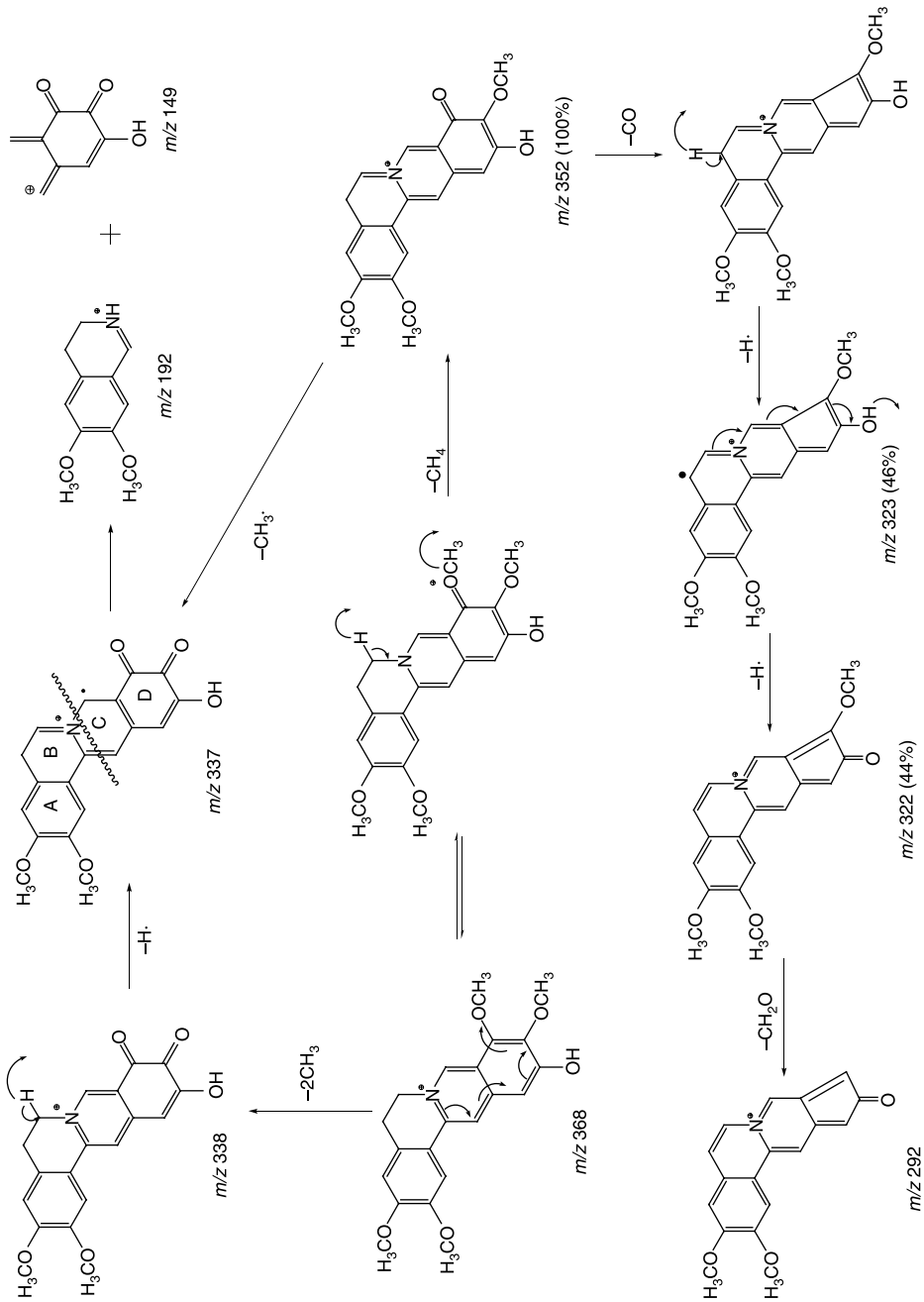


Figure 3. Proposed mass fragmentation of **4**.

specimen (GUH-17600) has been deposited in the departmental herbarium.

3.3 Extraction and isolation

Air-dried finely powdered tubers were extracted exhaustively with 95% ethanol at 30–50°C (for 15 h, three times) on a heating mantle. The extraction mixture was filtered and the solvent was evaporated up to dryness under reduced pressure to yield a black brown residue (200 g). It was chromatographed by pre-adsorbing onto silica gel (200 g) and then adding to the top of the column prepared using 500 g silica gel (60–120 mesh; Merck, Mumbai, India) in $CHCl_3$. Elution was first started with $CHCl_3$ and then with $CHCl_3$:MeOH (49:1, 24:1, 47:3, 23:2, 9:1, 22:3, 43:7, 21:4, and 41:9). The fractions obtained from the column were collected every 100 ml and combined on the basis of TLC analysis. The elution with $CHCl_3$:MeOH (9:1 and 22:3) was combined on the basis of TLC analysis. It was subjected to repeated column chromatography with 250 g silica gel and eluted with $CHCl_3$:MeOH (23:2, 91:9, 9:1, 89:11, 22:3, 87:13, and 43:7). The fractions from this column were collected for every 50 ml. The fractions from the elution of $CHCl_3$:MeOH (47:3 → 23:2) were further combined on the basis of TLC analysis, which showed five resolved spots along with some elongated spots with $CHCl_3$:MeOH (8:2). It was further subjected to repeated column chromatography using $CHCl_3$:MeOH (24:1, 19:1, 47:3, 93:7, 23:2, 91:9, and 9:1) as the eluting solvents and the fractions were collected for every 25 ml. The elution with $CHCl_3$:MeOH (19:1, 93:7, 23:2, and 91:9) furnished four quaternary protoberberine alkaloids as iodide salts named palmatine, dehydrocorydalmine, stepharanine, and 11-hydroxypalmatine, respectively, whereas the fifth compound shown by TLC could not be separated.

3.4 11-Hydroxypalmatine (4)

Yellow needles (MeOH, 360 mg); mp 228–230°C; M.F. $C_{21}H_{22}NO_5I$; UV: λ_{max}^{MeOH} 285, 253, 213 nm; IR: ν_{max}^{KBr} 3508,

Table 1. ^{13}C, ^{1}H NMR, DEPT, and HMBC data of **4** in DMSO-d_6.

Position	δ_C	δ_H (J, Hz)	DEPT	HMBC
1	118.5	6.88 s	CH	C-3, C-4a
1a	123.1	–	C	–
2	151.5	–	C	–
3	149.9	–	C	–
4	108.3	7.57 s	CH	C-2, C-1a
4a	121.3	–	C	–
5	26.3	2.5 t (2.4, 2.4)	CH_2	C-1a
6	61.5	4.92 t (1.5, 1.5)	CH_2	C-13a, C-8, C-4a
8	133.1	8.79 s	CH	C-9, C-13a, C-12a
8a	126.2	–	C	–
9	144.8	–	C	–
10	143.6	–	C	–
11	148.8	–	C	–
12	110.6	7.95 s	CH	C-10, C-8a
12a	127.7	–	C	–
13	120.0	7.78 s	CH	C-8a, C-1a
13a	137.6	–	C	–
OMe-2	55.4	3.88 s	CH_3	C-2
OMe-3	55.7	4.03 s	CH_3	C-3
OMe-9	56.0	4.12 s	CH_3	C-9
OMe-10	56.6	3.94 s	CH_3	C-10
OH-11	–	9.81 s	–	C-10, C-12

3236, 1651, 1511, 1402 cm^{-1}; ^1H, ^{13}C NMR, DEPT, and HMBC data: see Table 1; LC-EI-MS (rel. abun.): *m/z* 495 $[M]^+$ (5), 368 (13), 352 (100), 338 (84), 206 (14), 192 (10), 149 (5); Elemental analysis: found C, 51.20; H, 4.42; I, 25.46; N, 2.82; O, 16.10, calcd for $C_{21}H_{22}NO_5I$: C, 50.92; H, 4.48; I, 25.62; N, 2.83; O, 16.15%.

3.5 Silver nitrate test

A mixture of **4** (5 mg) and 2% HNO_3 (5 ml) was heated up to boiling temperature. After cooling, 10 ml of $AgNO_3$ was added to it. A deep yellow precipitate (insoluble in NH_4OH) was obtained, which was suggestive of an iodide salt.

3.6 Reaction with zinc dust

Zn dust (2 mg) was added to compound **4** (5 mg) in MeOH and heated for 15 min, afforded a yellow color compound on cooling, recrystallized with MeOH. This was identified as palmatine by direct comparison (UV, IR, co-TLC, mmp.) with an authentic sample.

3.7 Hypoglycemic activity

3.7.1 Preparation of doses

The oral doses of **4** at 25, 50, and 100 mg/kg, p.o., body weight were prepared in distilled water for the determination of the hypoglycemic effect, whereas the oral doses of 100, 200, 300, 400, and 500 mg/kg, p.o., were prepared for LD_{50} experiments. Glibenclamide (as standard) 5 mg/kg, p.o., was prepared with distilled water (Table 2).

3.7.2 Study of the test compound and positive control on experimental animals

Swiss albino mice of either sex (35–50 g body weight) were employed for the present study. These animals were deprived of food for 16 h but were allowed free access to water. They were housed in the departmental animal house and exposed to normal light. Experiments were performed according to the guide for the care and use of laboratory animals, from the CPCSEA, Ministry of Environment and Forest, Government of India (Reg. No. 107/1999/CPCSEA). After deprived of food for 16 h, mice were divided into six groups (six animals each), I–VI, namely normal control, diabetic control, diabetes+HP-25, diabetes+HP-50, diabetes+HP-100 mg/kg, and positive control. Induction of diabetes was performed using a modification in the method described by Shan *et al.* [14]. Diabetes was produced by an injection of alloxan (60 mg/kg, dissolved in saline) in the tail vein of mice. The diabetic state was assessed by blood glucose levels 36 h after alloxan administration, and the mice

Table 2. Hypoglycemic effects of different doses of **4**.

Time (h)	Diabetic control[a] ± SD	Normal control ± SD	Diabetic+ HP-25[b] ± SD	Diabetic+ HP-50[b] ± SD	Diabetic+ HP-100[b] ± SD	Positive control ± SD
0	192 ± 1.7	103 ± 2.3	187 ± 2.3	179 ± 1.1	189 ± 1.9	181 ± 0.8
1	181 ± 1.4	99 ± 0.5	163 ± 2.0	159 ± 1.3	169 ± 1.5	176 ± 2.1
3	186 ± 0.5	92 ± 0.6	151 ± 1.3	138 ± 1.8	131 ± 1.8	158 ± 2.0
6	160 ± 2.1	89 ± 1.3	164 ± 1.8	145 ± 1.2	140 ± 0.5	128 ± 2.2
18	157 ± 1.9	84 ± 2.1	152 ± 0.5	128 ± 1.4	102 ± 0.7	116 ± 1.7
24	139 ± 0.8	81 ± 1.7	129 ± 2.1	114 ± 2.2	90 ± 2.1	82 ± 1.7
Total Reduction	27.6%	21.36%	31.03%	36.31%	52.38%	54.69%

Notes: Values are mean ± SEM for six animals. SD, standard deviation; HP, 11-hydroxypalmatine.
[a] $p < 0.05$ vs. normal control.
[b] $p < 0.05$ vs. positive control.

having blood glucose more than 150 mg/dl were only selected for the study. Animals which presented glucose levels lower than 150 mg/dl were rejected. The normal control group (I) was not administered by alloxan and only received distilled water. The rest of the groups (II–VI) received alloxan and 36 h later were treated with distilled water (diabetic control), groups III–V with 25, 50, and 100 mg/kg, p.o., respectively, of **4**, and group VI was treated with 5 mg/kg glibenclamide, p.o., as standard. Blood samples of normal and alloxan-induced diabetic mice were collected at 0, 1, 3, 6, 18, and 24 h during the treatment. In each case, 10 μl of the serum sample was collected and estimated for glucose by the glucose oxidase–peroxidase method [15].

3.7.3 LD_{50} experiment

Mice were administered **4**, orally at doses of 100, 200, 300, 400, and 500 mg/kg, p.o., body weight and observed continuously for 1 h intermittently up to 24 h for any gross behavioral changes and death.

3.7.4 Data and statistical analysis

Results are expressed as the mean ± SEM of six independent experiments. The data were analyzed for statistical significance by one-way ANOVA test; p values < 0.05 were considered to be significant.

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