

Chemical and Antiviral Study on Alkaloids from *Papaver pseudocanescens* M. Pop.

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The phytochemical investigation of the aerial parts of *Papaver pseudocanescens* M. Pop. of Mongolian origin resulted in the isolation and structural elucidation of 8 alkaloids of the isoquinoline and promorphinane type. 8,14-Dihydroamurine, 8,14-dihydroflavinantine, and flavinantine are promorphinanes. Alborine, mecambidine, and mecambidine methohydroxide are retroprotoberberines. Amurensinine is an isopavine alkaloid and *O*-methyarmepavine is a benzylisoquinoline alkaloid. *O*-Methylarmepavine is a new alkaloid for the genus *Papaver*. Promorphinane-type alkaloids have been found for the first time in the species. All structures were established by physical and spectral analysis. As a first attempt to describe some of the biological activities of these alkaloids, the antiviral effect was tested against the *in vitro* replication of several viruses which belong to different taxonomic groups and represent significant human pathogens. Based on the results, the conclusion could be drawn that particular alkaloids from *P. pseudocanescens* possess selective antiviral effects against the replication of poliovirus 1 and human rhinovirus 14, two viruses from the *Enterovirus* genus of the Picornaviridae family.

Key words: *Papaver pseudocanescens* M. Pop., Alkaloids, Antiviral Activity

Introduction

The Papaveraceae represent a large family divided into 23 genera with more than 300 species, distributed world-wide in subtropical and temperate regions (Reveal, 1999). The genus *Papaver* L. is represented by more than 120 species, rich in isoquinoline and morphinane alkaloids (Preininger, 1986). In our previous phytochemical studies on *Papaver nudicaule* L. growing in Mongolia, we reported the isolation and identification of 13 alkaloids of the promorphinane, proaporphine, protopine, and isopavine type, respectively (Philipov *et al.*, 2007; Istatkova *et al.*, 2008). As a continuation of our investigations on Mongolian alkaloid-containing plants belonging to the genus *Papaver*, our attention turned to *Papaver pseudocanescens* M. Pop. This species is spread over the Khangai, Mongol Altai, and Gobi Altai regions of Central Mongolia (Grubov, 1982). There are data about

the use of this species in Mongolian folk medicine as a sedative, antitussive, and respiratory regulative remedy, as well as against headache caused by nervous disorders, against painful menstruation, and acute and chronic inflammation of the stomach (Ligaa, 1996). Chemical studies on this species have been reported, and several alkaloids of the retroprotoberberine, isopavine, protopine, rhoeadine, and papaverrubine type, respectively, have been isolated (Novák and Slavík, 1974; Preininger, 1986). To our knowledge no phytochemical investigations of *P. pseudocanescens* growing in Mongolia have been carried out till now.

Alkaloids are a rich source of biologically active products and some of them have been tested for antiviral activity (Hudson, 1990). Isoquinoline alkaloids were found to inhibit selectively parainfluenza virus 3 *in vitro* (Orhana *et al.*, 2007). Some of the alkaloids, isolated from the plants of the Papaveraceae family, are very important in medi-

cine, and some of them are considered as promising ones because of their antiviral activities against the replication of poliovirus, encephalomyocarditis virus, influenza virus, herpes simplex virus, and human adenovirus 5 and 12 (Colombo and Bosisio, 1996). Aporphine alkaloids have been reported to possess antiviral activity against the replication of poliovirus 1 (Boustie *et al.*, 1998) and herpes simplex virus type 1 (Montanha *et al.*, 1995).

The aim of the present work was to determine the alkaloid composition of *P. pseudocanescens* of Mongolian origin as well as to conduct antiviral tests as a first attempt to describe the biological activities of these alkaloids. Eight alkaloids of the promorphinane, retroprotoberberine, isopavine, and benzyloquinoline type, respectively, have been isolated from the aerial parts and determined by spectral data. The benzyloquinoline alkaloid **7** is new for the genus *Papaver* and the promorphinanes **1**, **4**, and **5** are new type alkaloids for the species.

Material and Methods

General

UV spectra were measured with a SESIL (Cambridge, England) CE 8020 instrument, in MeOH. IR spectra were taken on a Bruker (Ettlingen, Germany) IFS113 V instrument, in KBr. EI-MS was performed on a Hewlett Packard (Palo Alto, CA, USA) MSD 5973 instrument, at 70 eV. ¹H NMR spectra were recorded on a Bruker (Fällanden, Switzerland) DRX-250 instrument, in CDCl₃, with TMS as an internal standard. Vacuum liquid chromatography (VLC) was carried out on silica gel (Merck, Darmstadt, Germany) and Kieselgel 60 (70 – 230 mesh). Column chromatography (CC) was carried out on neutral alumina (Merck; aluminium oxid 90, act. II–III Brockmann, 70–230 mesh). Preparative thin layer chromatography (PTLC) was run on 20 x 20 cm plates (0.5 mm thickness) with Kieselgel 60 GF₂₅₄ (Merck). The mobile phases (Mph) used for PTLC were petroleum ether/CHCl₃/Me₂CO/MeOH: Mph1, 4:4:1:1, v/v/v/v; Mph2, 4:8:1:2, and Mph3, 2:8:1:3. Thin layer chromatography (TLC) was performed on plates with Kieselgel 60 F₂₅₄ DC-Alufolien (Merck), and spots were detected under UV light. Visualization for TLC was done with Dragendorff's reagent.

Plant material

The aerial parts of *P. pseudocanescens* were collected in August 2005 during the time of flowering, in the Zagastain davaa Mountain, Zarkhan province, Central Mongolia. A voucher specimen (No. 5248) is deposited in the Herbarium Fund of the Institute of Botany, Mongolian Academy of Sciences (Ulaanbaatar, Mongolia). The plant material was identified by Prof. E. Ganbold, Institute of Botany, Mongolian Academy of Sciences.

Extraction and isolation

Air-dried and ground aerial parts (3.8 kg) were extracted exhaustively with 95% EtOH at room temperature. The combined EtOH extracts were evaporated under reduced pressure, acidified with 5% HCl (300 ml), and left overnight at room temperature. Insoluble non-alkaloid materials were removed by filtration, and the filtrate was subjected to *n*-hexane extraction (6 x 200 ml) to eliminate the rest of the non-alkaloid substances. Thus the purified acidic solution was made alkaline to pH 9–10 with 25% NH₄OH and extracted with CHCl₃ (6 x 300 ml). The combined CHCl₃ extracts were dried (anhydrous Na₂SO₄) and evaporated under reduced pressure to give 2.50 g crude mixture of tertiary alkaloids.

The crude alkaloid mixture was further separated by VLC on silica gel, eluted with CHCl₃/MeOH of increasing polarity (pure CHCl₃, 99:1, 97:3, 95:5, 90:10, 70:30, and pure MeOH), and 8 combined alkaloid fractions (AF1 to AF8) were obtained.

AF1 (CHCl₃; 0.28 g) was worked up by CC on neutral alumina, eluted with *n*-hexane/EtOAc of increasing polarity (10:1, 9:1, 7:1, 5:1, 3:1, 1:1, and pure EtOAc) to afford 6 combined fractions (AF1-1 to AF1-6) enriched in individual alkaloids. In AF1-1 (10:1; 53.66 mg) no alkaloids were detected. AF1-2 (10:1; 64.20 mg) was subjected to PTLC with Mph1 as mobile phase, and alkaloid **1** (8.00 mg) was isolated. AF1-3 (9:1, 7:1; 53.95 mg) was subjected to PTLC with Mph1 as mobile phase, and alkaloid **2** (35.97 mg) was isolated. The area around the start of the plate was extracted, evaporated to dryness, and then subjected to PTLC with Mph3 as mobile phase, and alkaloid **3** (4.84 mg) was isolated. AF1-4 (5:1; 30.77 mg) was subjected to PTLC with Mph1 as mobile phase, and alkaloid **4** (4.88 mg) was isolated. Fractions AF1-5 (3:1) and AF1-6 (1:1, EtOAc) were ob-

tained in insufficient quantities (each less than 10 mg) for further isolation and characterization of pure compounds.

AF2 (CHCl₃; 0.79 g) was worked up by CC on neutral alumina, eluted with *n*-hexane/EtOAc of increasing polarity (7:1, 5:1, 3:1, 1:1, and pure EtOAc) to afford 4 combined fractions (AF2-1 to AF2-4) enriched in individual alkaloids. AF2-1 (7:1; 18.66 mg) was subjected to PTLC with Mph1 as mobile phase, and alkaloid **5** (5.50 mg) was isolated. AF2-2 (5:1; 113.00 mg) was subjected to PTLC with Mph2 as mobile phase, and alkaloid **6** (17.57 mg) was isolated. AF2-3 (3:1; 478.00 mg) (100 mg from it) was subjected to PTLC with Mph1 as mobile phase, and alkaloid **2** (63.90 mg) was isolated. The area around the start of the plate was extracted, evaporated to dryness, and then subjected to PTLC with Mph3 as mobile phase, and alkaloid **3** (6.50 mg) was isolated. In AF2-4 (1:1, EtOAc; 100.33 mg) no alkaloids were detected.

AF3 (99:1; 0.13 g) and AF4 (99:1; 0.16 g) were separately subjected to PTLC with Mph1 as mobile phase, and alkaloid **5** (24.24 mg from AF3 and 14.12 mg from AF4) was isolated.

AF5 (97:3; 0.04 g) was directly subjected to PTLC with Mph3 as mobile phase, and alkaloid **3** (24.90 mg) was isolated.

AF6 (97:3; 0.08 g) and AF7 (95:5, 90:10, 70:30; 0.19 g) were separately subjected to PTLC with Mph3 as mobile phase and alkaloids **3** (4.30 mg from AF6 and 16.00 mg from AF7), **7** (2.16 mg from AF6 and 5.70 mg from AF7), and **8** (18.34 mg from AF6 and 45.34 mg from AF7) were isolated.

AF8 (MeOH; 0.17 g) was directly subjected to PTLC with Mph3 as mobile phase and alkaloid **8** (39.00 mg) was isolated.

Screening for antiviral activity

Compounds

The alkaloids were solubilized and kept as 20-mM stock solutions in dimethyl sulfoxide (DMSO). Stock solutions were further diluted to the desired working concentrations in a maintenance medium. Oxoglucine, an aporphinoid alkaloid from *Glaucium flavum* Cranz, and disoxaril (WIN 51 711, 5-[7-(4,5-dihydro-2-oxazolyl)-phenoxyheptyl]methylisoxazole), supplied by Sanoft Winthrop Inc. (Malverne, PA, USA), served as reference anticornavirus compounds. The reference compound for human respiratory syncytial

virus and human adenovirus was ribavirin (1- β -D-ribofuranosyl-1*H*-1,2,4-triazole-3-carboxamide), a kind gift from Prof. Robert W. Sidwell, Logan, USA. Ribavirin was dissolved directly to the required concentrations in the maintenance medium.

Cells and viruses

Poliovirus type 1 (LSc-2ab), coxsackievirus B1 (CV-B1), human rhinovirus type 14 (HRV-14), human respiratory syncytial virus A2 (HRSV-A2), and human adenovirus 5 (HAdV-5), all from the cell culture collection of the Stephan Angeloff Institute of Microbiology of the Bulgarian Academy of Sciences (Sofia, Bulgaria) were used for the antiviral tests. LSc-2ab, CV-B1, and HAdV-5 were grown in the FL cell line. HRV-14 and HRSV-A2 were grown in HeLa Ohio-1 cells and HEP-2 cells, respectively. The cells were also from the cell culture collection of the Stephan Angeloff Institute, HeLa Ohio-1 cells being a recent kind gift from Dr. Dale Barnard, Utah State University, Logan, USA. All cell lines were grown in a humidified atmosphere at 37 °C and 5% CO₂ in Dulbecco modified Eagles' medium (DMEM) (Gibco BRL, Grand Island, NY, USA), except for HeLa Ohio-1 cells, which were propagated in minimal essential medium (MEM) (Gibco BRL). The growth medium contained 5% fetal bovine serum in the case of FL and HEP-2 cells, and 10% fetal bovine serum for HeLa Ohio-1 cells, supplemented with antibiotics (100 IU/ml penicillin, 100 μ g/ml streptomycin, and 50 μ g/ml gentamycin), and 20 mM HEPES buffer (Gibco BRL), the latter providing supplemental buffering to cell culture medium at pH 7.2 through 7.6. Cells were routinely subcultured twice weekly. When harvesting viruses and performing antiviral assays, a maintenance medium was used, in which serum was reduced to 2% for HRV-14 and to 0.5% for the remaining viruses. Viruses were grown in a humidified atmosphere at 37 °C and 5% CO₂ with the exception of HRV-14, which was grown at 33 °C.

Cellular toxicity

Monolayer cells in 96-well plates (Cellstar[®], Greiner Bio-one GmbH, Frickenhausen, Germany) were inoculated with 0.1 ml/well containing concentrations (in 0.5 lg intervals) of the compounds diluted in a maintenance medium. After 24 h of incubation in a humidified atmosphere at 37 °C and 5% CO₂, cells were monitored

for microscopic cytotoxic effects and the highest concentration, at which no visible cytotoxic effect was recorded, was considered as the maximal tolerated concentration (MTC). After the microscopic evaluation, cells were subjected to the neutral red-uptake procedure (Borenfreund and Puernen, 1985; Repetto *et al.*, 2008), and the 50% cytotoxic concentration (CC_{50}) was calculated. Briefly, after removal of the maintenance medium containing the test compound, cells were washed and 0.1 ml fresh maintenance medium containing 0.005% neutral red dye (Fluka Chemie AG, Buchs, Switzerland) was added to each well; cells were incubated at 37 °C for 3 h. Then cells were washed once with phosphate-buffered saline (PBS), and 0.15 ml/well desorb solution (1% glacial acetic acid, 49% ethanol, 50% distilled water) was added. Following 10 min of mild shaking, the optical density (OD) of each well was read at 540 nm in a microplate reader (Organon Teknika reader 530; Oss, Netherlands). The CC_{50} value was defined as the concentration of each compound that reduced the absorbance of treated cells by 50% when compared to the untreated control. The CC_{50} values were determined by regression analysis.

Antiviral activity

The virus cytopathic effect (CPE) inhibition assay was used for evaluating the antiviral effects of the alkaloids. Monolayer cells in 96-well plates were inoculated with 0.1 ml virus suspension containing 100 $CCID_{50}$ (1000 $CCID_{50}$ in the case of HRV-14). $CCID_{50}$ is the cell culture infectious dose 50% which was previously determined by the standard virus titration assay in the respective cell culture. Mock-infected wells were left for toxicity and cell controls. After 1 h of virus adsorption (2 h in the case of HRSV-A2 and HRV-14), excessive virus was discarded, and cells were inoculated with 0.2 ml of maintenance medium containing non-toxic concentrations (in 0.5 lg intervals) of the test compounds. Then cells were further incubated in a humidified atmosphere at 37 °C (33 °C in the case of HRV-14) and 5% CO_2 . The CPE was scored daily till the appearance of its maximum in the virus control wells (with no compound in the maintenance medium). Then viable cells were stained according to the neutral red-uptake procedure, and the percentage of CPE inhibition for each concentration of the test compound was calculated using the follow-

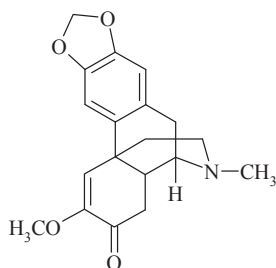
ing formula: $\% \text{ CPE} = \frac{[OD_{\text{test sample}} - OD_{\text{virus control}}]}{[OD_{\text{toxicity control}} - OD_{\text{virus control}}]} \cdot 100$, where $OD_{\text{test sample}}$ is the mean value of the OD values of the wells inoculated with virus and treated with the test compound in the respective concentration, $OD_{\text{virus control}}$ is the mean value of the OD values of the virus control wells (with no compound in the medium), and $OD_{\text{toxicity control}}$ is the mean value of the OD values of the wells not inoculated with virus but treated with the corresponding concentration of the test compound. The concentrations that inhibited 50% of the virus induced CPE, the 50% inhibitory concentrations (IC_{50}), were determined by regression analysis. The selectivity index (SI) was calculated as the ratio of CC_{50} and IC_{50} ($SI = CC_{50}/IC_{50}$). When it was not possible to calculate the IC_{50} values due to a minor but real antiviral effect, results were expressed as approximate percentages of viral inhibition.

Results and Discussion

Phytochemical study

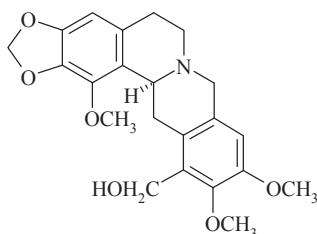
The phytochemical investigation of the aerial parts *P. pseudocanescens* growing in Mongolia afforded 8 alkaloids of the promorphinane, retroprotoberberine, isopavine, and benzyloquinoline type, respectively. Three of the alkaloids are promorphinanes (**1**, **4**, **5**), three are retroprotoberberines (**2**, **3**, **8**), one is of the isopavine type (**6**), and one is of the benzyloquinoline type (**7**). The chemical structures of these alkaloids are presented in Fig. 1. Compounds **1**, **4**, **5**, and **6** were identified by comparison of their UV, IR, EI-mass, and 1H NMR spectral data with those of authentic samples originating from other plant material (Philipov *et al.*, 2007; Istatkova *et al.*, 2008). The data of the provided UV, EI-mass, and 1H NMR spectra for compounds **2**, **3**, and **8** are in a good agreement with the literature data (Pfeifer *et al.*, 1967; Preininger *et al.*, 1969, 1970). The structure of alkaloid **7** was determined by comparison of its EI-mass and 1H NMR spectra with the literature data (Tomita *et al.*, 1966; Shamma, 1972).

The benzyloquinoline alkaloid **7** has been found for the first time in the genus *Papaver*. The promorphinane alkaloids **1**, **4**, and **5** are new for the species. This is the first case that the presence of promorphinane-type alkaloids in *P. pseudocanescens* is reported.

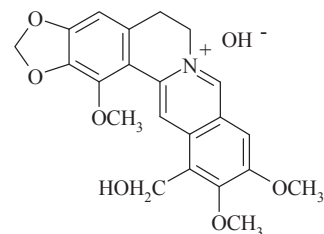


Racem. 8,14-dihydroamurine (1)

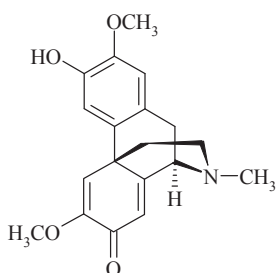
(new for the species)



(-)-Mecambridine (2)

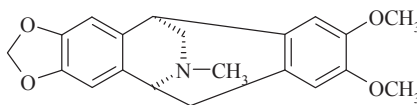


Alborine (3)

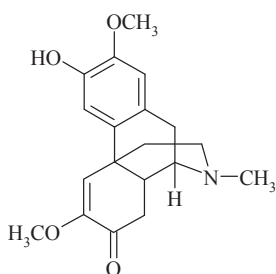


(-)-Flavinantine (5)

(new for the species)

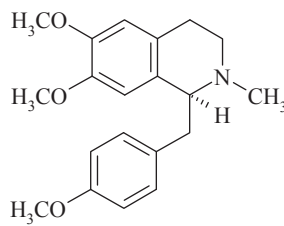


(-)-Amurensinine (6)



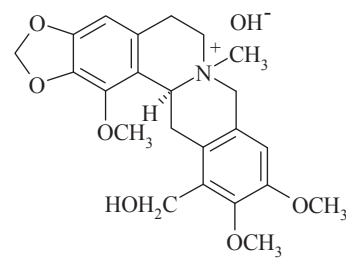
Racem. 8,14-dihydroflavinantine (4)

(new for the species)



(+) -O-Methylarmepavine (7)

(new for the genus)



(-)-Mecambridine methohydroxide (8)

Fig. 1. Chemical structures of the alkaloids.

Biological assay

The alkaloids were tested for their antiviral activity against viruses representing important human pathogens from different taxonomic groups, *i.e.* Picornaviridae, Paramyxoviridae, and Adenoviridae. To date no selective antiviral drug is registered for the treatment of human infections by

picorna- and adenoviruses. To our knowledge no antiviral activity has been reported for the alkaloids reported here. The antiviral activity of the isolated alkaloids was tested by the multi-cycle CPE inhibition test at a standard virus dose of 100 CCID₅₀/well (1000 CCID₅₀ in the case of HRV-14). The following antiviral compounds were in-

cluded as positive controls because of their markedly expressed specific antiviral effect against the corresponding viruses: disoxaril and oxoglaucine (Nikolaeva-Glomb *et al.*, 2008) for poliovirus 1 and HRV-14, and ribavirin for HRSV-A2 and HAdV-5. The tested alkaloids demonstrated differential antiviral activity depending on the respective virus and the alkaloid. The replication of

human respiratory syncytial virus A2 and human adenovirus 5 was not affected by the alkaloids (data not shown). On the contrary, particular compounds exhibited promising antiviral activity against the replication of two of the enteroviruses included in the test panel, namely poliovirus type 1 and human rhinovirus type 14. Values obtained for the cytotoxicity on FL and HeLa Ohio-1 cells

Table I. *In vitro* antiviral effects of alkaloids from *Papaver pseudocanescens* M. Pop. against poliovirus 1 and human rhinovirus 14.

Compound	Cytotoxicity CC ₅₀ [μM] ^a		CPE inhibition					
			Poliovirus 1		Human rhinovirus 14			
	FL cells	HeLa cells	IC ₅₀ [μM] ^a	%CPE ^b	SI	IC ₅₀ [μM] ^a	%CPE ^b	SI
1	37 ± 4.24	166 ± 5.66	- ^c	≥ 50	-	-	≥ 50	-
2	139 ± 4.24	174 ± 8.48	-	≤ 25	-	-	-	-
3	190 ± 28.28	> 200	49.7 ± 2.26	≈100	3.84	-	-	-
4	108 ± 9.89	373 ± 38.18	-	< 10	-	199 ± 15.56	> 75	1.87
5	136 ± 5.65	207 ± 11.31	-	< 30	-	65 ± 7.07	≈100	3.18
6	49 ± 1.41	273 ± 38.18	-	≥ 50	-	-	-	-
7	123 ± 7.07	151 ± 12.73	-	≤ 25	-	-	-	-
8	188 ± 16.97	> 200	21.4 ± 3.68	100	8.79	-	-	-
Oxoglaucine	52 ± 7.07	60 ± 4.24	1.2 ± 0.11	100	43.3	0.3 ± 0.14	100	200
Disoxaril	25 ± 4.24	21 ± 5.66	2 ± 1.13	100	12.5	1.5 ± 0.28	100	14

^a CC₅₀ and IC₅₀ values with SD values shown represent the mean values of two different experiments with four replicates in each experiment.

^b Percentage of virus cytopathic effect inhibition at the highest nontoxic concentration tested as compared to the non-infected control.

^c Could not be determined or not applicable.

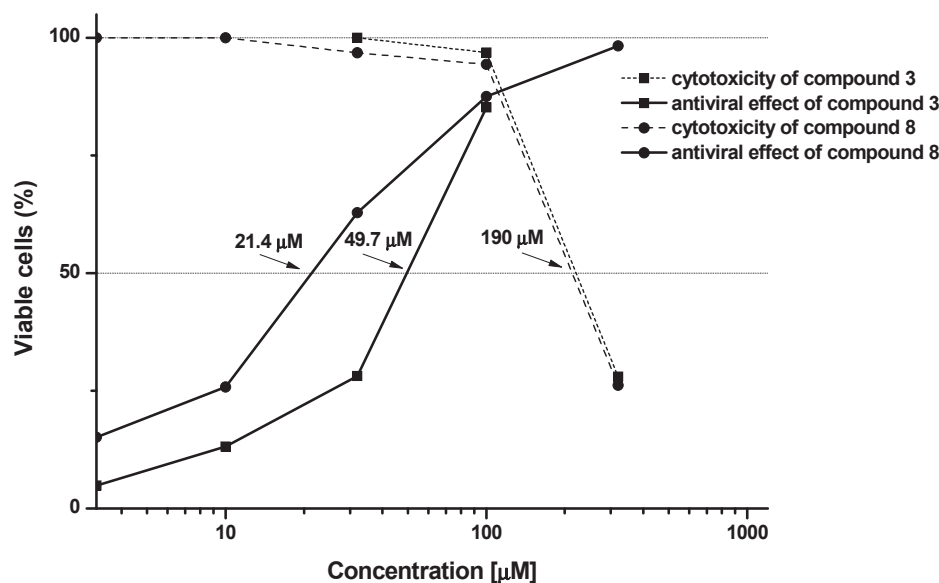


Fig. 2. Effect of alborine (**3**) and mecambidine methoxyhydroxide (**8**) on the *in vitro* replication of poliovirus 1 (LSc-2ab) in monolayer FL cells in the CPE-inhibition experimental set-up (dose-response curves). (Dashed lines represent the dose-response curves of the cytotoxicity controls.) Arrows indicate IC₅₀ and CC₅₀ values.

(50% cytotoxic concentration, CC_{50}), CPE inhibition (50% inhibitory concentration, IC_{50}), and selectivity indices (SI, expressed as the ratio between CC_{50} and IC_{50}) are presented in Table I. Cocksackievirus B1, the third enterovirus tested, was not inhibited by the alkaloids.

The results reveal that all alkaloids, to a different extent, are active against the replication of poliovirus type 1. **8** exerts the most promising effect with a selectivity index exceeding 8. Another retroprotoberberine, *i.e.* **3**, also possesses a noticeable activity, although a slighter one with $SI \approx 4$. The dose-response curves of compounds **3** and **8** demonstrate the significant and dose-dependent character of the antiviral effect (Fig. 2).

Interestingly, the three retroprotoberberines, although active against poliovirus type 1, are unable to inhibit the replication of the other two tested enteroviruses, CV-B1 and HRV-14. The replication of CV-B1 was not affected by any of the tested alkaloids, but human rhinovirus type 14 was inhibited by the promorphinanes, and among them **5** was the most active with a selectivity index above 3. The three alkaloids active against HRV-14 replication were those newly described for *P. pseudocanescens*.

Given the promise of the above results, the alkaloids isolated from *P. pseudocanescens* deserve further attention and detailed research as promising antiviral compounds.

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