SHORT REPORT

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Cytotoxic Activity of Alkaloids from *Papaver rhoeas* growing in Lebanon

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Abstract: This paper represents the phytochemical properties of Lebanese *Papaver rhoeas*, from which five protopine alkaloids are isolated, namely; stylopine (**1**), canadine (**2**), sinactine (**3**), berberine (**4**), and epiberberine (**5**). This is the first report for the isolation of epiberberine (**5**) from the genus Papaver and canadine (**2**) from *Papaver rhoeas*, suggesting a new chemotype of *Papaver rhoeas* growing in Lebanon. The cytotoxic activity of the total ethanolic extract and the isolated alkaloids were determined by MTT assay on human colon cancer cells (HCT116), breast cancer cells (MCF7), human keratinocyte cell line (HaCaT), and non-cancerous colon cells (NCM460). The compounds showed dose-dependent inhibitory effect with highest activity for compound **4** against all cell lines. The activity of the alkaloids varied between the various cell lines indicating cell type specificity and suggesting different cell-compound interactions. IC_{50} values on normal cells was higher than cancer cell lines (>200 µM), indicating the selectivity of these compounds to cancer cells. It was noticed that the presence of methylenedioxy group at positions C-2 and C-3 rather than at position C-9 and C-10 potentiated the compound's cytotoxic activity. Further studies are underway to explore the activity of these compounds at the molecular level.

Keywords: Cytotoxic activity; *Papaver rhoeas*; protopine alkaloids; spectroscopic analysis.

1. Plant source

Aerial parts of *Papaver rhoeas* were collected, during the flowering period in April 2014, from west Beqaa region about 1300 m above sea level. The plant was authenticated by Dr. George Tohme, Professor of Taxonomy (National Council for Scientific Research (CNRS), Beirut, Lebanon). A voucher specimen (pr-16-14) was deposited in the herbarium of the Faculty of Pharmacy, Beirut Arab University, Beirut, Lebanon. The plant was dried under shade at room temperature, and the dried aerial parts were ground into moderately coarse powder**.**

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2. Previous Studies

Plants of Papaveraceae have the capacity to synthesize various biologically active and structurally complex alkaloids that are not easily and economically synthesized [1]. These alkaloids are the most pharmacologically important compounds especially the isoquinoline type [2-4]. Papaver alkaloids are well known for their antioxidant, antimutagenic, anticarcinogenic [5-7], antimicrobial [8], anti-inflammatory and antifibrotic activities [9]. They might be also considered as potential therapy for hypercholesterolemia [10], diabetes, and related diseases [11-12]. *P. rhoeas* extract proved to be useful additive in skin cosmetic and pharmaceutical preparations that are intended to increase the rate of hyaluronic acid [13]. It has been used also for the treatment of diarrhea, cough, analgesia and the reduction of withdrawal signs of the opioid addiction [14]. The plant was also reported to inhibit ulcer in rats [15] and morphine tolerance in mice [16].

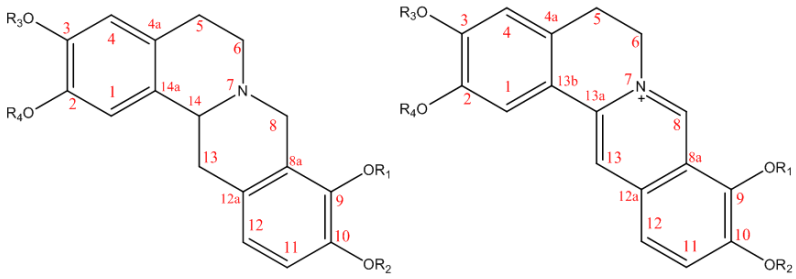
3. Present Study

The powdered aerial parts (4kg) were extracted with ethanol 96% (10 L x 4) at laboratory temperature. The alcoholic extract was concentrated under reduced pressure at 40-50 °C and then lyophilized to get powders. The lyophilized powder was extracted with 10% sulphuric acid (250 x 4 ml), the combined filtered acidic extract was washed with diethyl ether (300 x 2 ml) and the washings was discarded. The aqueous extract was rendered alkaline with concentrated ammonium hydroxide (NH_4OH) solution. The alkaline aqueous solution was then extracted with chloroform ($CHCl_3$, 6 x 300 ml). The combined chloroformic extracts were washed with water (300 ml), dried over anhydrous sodium sulphate, filtered and concentrated to dryness under reduced pressure to yield 4g of total crude alkaloids (0.1 % yield).

Chromatographic Isolation of Compounds: The total alkaloids extract was fractionated over a column chromatography (CC) using neutral alumina (400 g, column diameter 3.5 cm). Elution was carried out with gradient mixtures of petroleum ether and ethyl acetate (PE: EtOAc, 30 to 100% EtOAc) and continued by EtOAc and methanol (EtOAc: MeOH, 0 to 100% MeOH) to give 200 fractions each 150ml. Each fraction was spotted on TLC, detected by UV lamp, and visualized by spraying with dragendorff's reagent. Fraction 1 (F1) was applied to preparative TLC (20 x 20 cm^2 glass plate, 0.5 mm thickness) to give compound 1. F2 and F3 were gathered and applied to preparative TLC (20 x 20 cm^2 glass plate, 0.5 mm thickness) to give compounds 2 and 3. The bands of compounds 1, 2, and 3 were detected using UV lamp, scratched, and then recovered by chloroform methanol mixture ($CHCl_3$: MeOH, 2:1) into the crystalline forms. Compounds 4 and 5 were crystallized from F142 and F145 respectively. All compounds were further purified by repeated crystallization from MeOH.

Cell Culture and Treatment: HCT116 $p53^{+/+}$ human colon cancer cells were kindly provided by Dr. Carlos Galmarini (Institut National de la Santé et de la Recherche Médicale, Lyon, France). The human breast cell lines MCF-7 (p53 wildtype, noninvasive) were obtained from ATCC. HCT116 $p53^{+/+}$ and MCF-7 were cultured in RPMI 1640 (Sigma-Aldrich®, UK) with 20mM HEPES and L-Glutamine. Media was supplemented with 1% Penicillin-Streptomycin (100 U/ml) and 10% heat-inactivated FBS (Sigma-Aldrich®, Germany). HaCaT immortal keratinocyte cell line from adult human skin, kindly provided by Dr. N. Darwiche (Department of Biochemistry, American University of Beirut), was cultured in DMEM containing 10% heat-inactivated fetal bovine serum, 1% sodium pyruvate, 1% penicillin–streptomycin and 1% kanamycin antibiotics with normal calcium concentration in the medium (2 mM). Human normal colon (NCM 460) cells were cultured in M3Base medium (INCELL CORPORATION, San Antonio, USA) containing growth supplements. Media was supplemented with 10% heat-inactivated FBS (Sigma-Aldrich, Germany). All cells were maintained at 37 ºC in a humidified atmosphere of 5% CO_2 and 95 % air. Unless otherwise mentioned, cells were seeded at 1.2 x 10^5 cells/ml and treated with ethanolic extract of *P. rhoeas* and the isolated compounds (**1**-**5**) at 50% confluency. The total ethanolic extract and the compounds were prepared in DMSO and the final DMSO concentration on cells was less than 0.3%.

Cell Viability Assays: Cells were plated in 96-well plates and treated with different concentrations of stylopine, canadine, sinactine, berberine, epiberberine, and the total ethanolic extract. The inhibition of cell growth was measured by the Cell Titer 96 non-radioactive cell proliferation kit (Promega Corp, Madison, Wisconsin, USA). The cell growth assay is an MTT based method that measures the ability of metabolically active cells to convert tetrazolium salt into a blue formazan product, and its absorbance is recorded at 595 nm. The IC_{50} represents the concentration at which 50% of the cells are viable.



1 $R_1+R_2= CH_2$, $R_3+R_4= CH_2$

2 $R_1=R_2= CH_3$, $R_3+R_4= CH_2$

3 $R_1+R_2= CH_2$, $R_3=R_4= CH_3$

4 $R_1=R_2= CH_3$, $R_3+R_4= CH_2$

5 $R_1+R_2= CH_2$, $R_3=R_4= CH_3$

Figure 1. Chemical structures of alkaloids **1-5**

Compound **1** was isolated as colorless crystals, mp 204-208°C, while compounds **2** and **3** were obtained as yellowish crystals with mp 163-169°C and 172-176°C respectively. ESI-MS afforded the positive ion at m/z 324.1 $[M+H]^+$ for compound **1**, and m/z 340.1 $[M+H]^+$ for compounds **2** and **3** in agreement with the molecular formula $C_{19}H_{17}O_4N$ and $C_{20}H_{21}O_4N$ respectively..Compounds **4** and **5** were isolated as bright yellow and orange crystals, mp 144-146°C and 258-262°C respectively. ESI-MS afforded the positive ion at m/z 336.1 $[M+H]^+$, indicating the molecular formula $C_{20}H_{18}O_4N$. Extensive analysis of the spectroscopic data (^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, HCCSOW, IR, MS, and UV spectra) and comparison with data available in the literature [17-20], led to the identification of five protopine alkaloids namely; stylopine (**1**), canadine (**2**), sinactine (**3**), berberine (**4**), and epiberberine (**5**) (Figure 1).

It is interesting to mention that the presence of compound **5** in genus Papaver and compound **2** in *Papaver rhoeas* is reported here for the first time. Epiberberine was isolated mostly from *Coptidis Rhizoma* [21]. All the isolated compounds belong to to isoquinoline class of alkaloids which exhibit the widest spectra of pharmacological activities [22] giving a special attention for the Lebanese Papaver as promising therapeutic agents for the treatment of many diseases. Moreover, the isoquinoline alkaloids are important components in chemical defense of the producing species [23-24], which might explain the different chemotypes of the Lebanese plants.

The results of the cytotoxic activity of the extract (*P. rhoeas*) and the five isolated compounds (**1-5**) after 24 h of treatment are summarized in Figure 2. Epiberberine and sinactine were reported to have no cytotoxic activity [25], while in this paper all compounds with the exception of sinactine showed dose-dependent inhibitory effect on the cancerous cell lines. The most effective of the five compounds was berberine, with IC_{50} of 90, 15, and 50 µM on HCT116, MCF7, and HaCaT cells, respectively (Table 1). Interestingly the crude ethanolic extract of *Papaver rhoeas* revealed promising cytotoxic activity (Table 1). especially against colon and breast cancer cells. The activity of each compound varied from cell type to other indicating cell type specificity and suggesting different interaction with the cells. IC_{50} values of the active compounds on non-cancerous cells (NCM460) was higher than cancer cell lines (>200 µM), indicating the selectivity of these compounds to cancer cells.

Comparing the cytotoxicity of the five alkaloids, it seems that methylenedioxy group at position C-2 and C-3 (2, 3-OCH_2O) rather than at position C-9 and C-10 (9, 10-OCH_2O) potentiate the compound's cytotoxic activity (berberine vs epiberberine, and canadine vs sinactine). Moreover, the unsaturation of the rings (C5-C6 and N7-C8) and not the tetrahydro compound structure (berberine vs canadine, and epiberberine vs sinactine) appear to increase the cytotoxic activity against HCT116 human colon cancer cell line. Similarly, the activity of berberine, stylopine, and canadine (with methylenedioxy group at position C-2 and C-3 rather than at position C-9 and C-10) was more intensified against HaCaT cells than epiberberine and sinactine. Only berberine was active against MCF7 cells (with very strong activity) followed by epiberberine, and to lesser extent canadine.

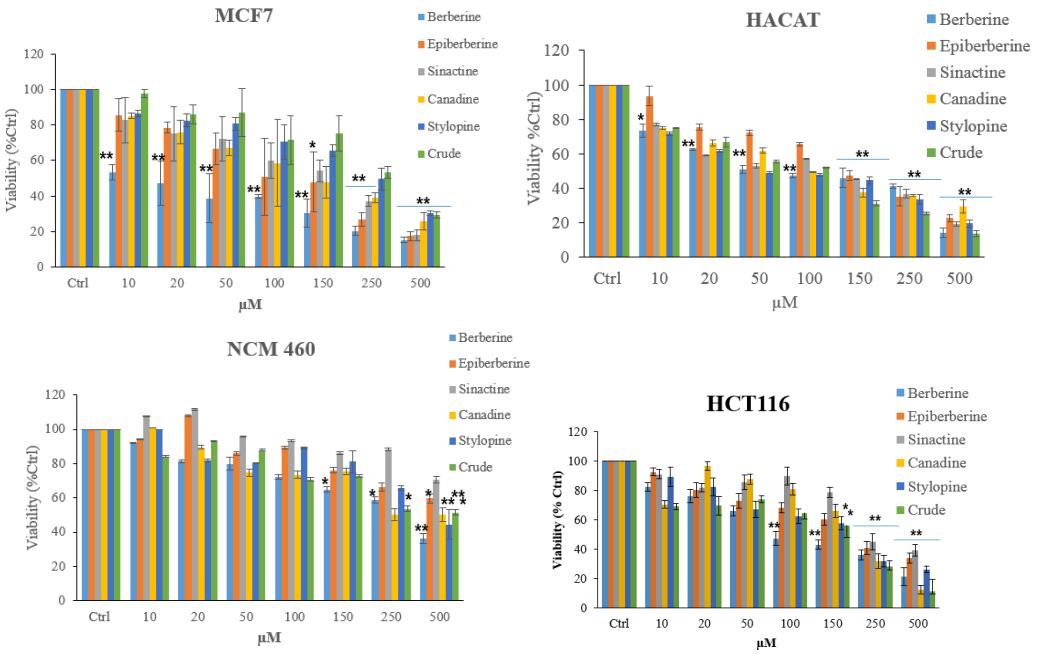


Figure 2. HCT116 p53+/+, MCF7, NCM460, and HaCaT cells were treated at 50% confluency with (stylopine, canadine, sinactine, berberine, epiberberine, and the total extract) (0, 10, 20, 50, 100,150, 250, and 500 µM) for 24h. Cell viability was determined by the MTT assay. Results are expressed as percentage of non-treated cells. Each value is the mean ±SD of three separate experiments each done in hexplicates. Results are expressed as percentage of non-treated cells. Each value is the mean ±SD of three separate experiments each done in hexplicates for statistical analysis *p < 0.05 and ** p<0.001, significant difference with respect to control.

Table 1. IC_{50} of compounds **1-5** and the total extract (µM))

Compound	**HCT116**	**MCF7**	**HaCaT**	**NCM460**
	IC_{50}±SD (µM)	**IC_{50}±SD (µM)**	**IC_{50}±SD (µM)**	**IC_{50}±SD (µM)**
Total extract	160 ± 4.41	>200	110±8.42	>200
Berberine	90± 3.39	15±2.61	50±5.12	>200
Stylopine	168 ± 4.86	>200	50±1.98	>200
Epiberberine	178 ± 2.75	125±3.15	140±2.43	>200
Canadine	>200	170±1.54	98±3.84	>200
Sinactine	>400	>200	150±2.85	>200

Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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