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# Cytotoxicity evaluation of natural coptisine and synthesis of coptisine from berberine☆

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**Abstract**

The crude extract (80% MeOH in water) of *Chelidonii herba* exhibited very interesting cytotoxicity against brine shrimp (*Artemia salina* Leach) nauplii and cultured human tumour cell in vitro, the colon carcinoma HT 29 (144 h treatment). Fractionation of the crude extract and bioassay-guided procedures showed that the cytotoxic and the antitumour activities were concentrated in the basic extract. On the basis of IR, MS and $^1$H NMR the compound responsible of the cytotoxic activity was determined to be coptisine. Cytotoxicity evaluation of coptisine was next extended to a panel of human and murine cell lines in comparison with the established antitumour drugs mitoxantrone, doxorubicin (Dx) and cisplatin (CDDP). Coptisine was cytotoxic on LoVo and HT 29 and less potent on L-1210, and it was partially crossresistant on the human tumour colon cell line resistant to Dx, LoVo/Dx, whereas it was not significantly crossresistant on the murine leukaemia cell line resistant to CDDP, L-1210/CDDP. Coptisine alkaloid was then synthesised in gram amount from commercial berberine. A four-step synthetic route was elaborated. The overall yield was about 8–10%. The structural identity of synthetic coptisine was verified by IR and NMR methods. A comparison of the cytotoxic effects on the human tumour colon cell line LoVo and on the murine leukaemia L1210 showed, for both natural and synthetic coptisines, a comparable cytotoxic activity more evident against HT 29 cell line and LoVo cell line, while the activity was lower against the L1210 cell line. © 2001 Éditions scientifiques et médicales Elsevier SAS

*Keywords:* *Chelidonium majus*; Protoberberine alkaloids; Coptisine; Synthesis; Cytotoxicity

## 1. Introduction

In a program of evaluation of the antiproliferative activity of plants, a large number of extracts from higher plants, mainly belonging to the mediterranean flora, were tested. A preliminary screening was performed against brine shrimp (*Artemia salina* Leach.) nauplii and a cultured human tumour cell in vitro, the colon carcinoma HT 29.

Among the tested plants, *Chelidonium majus* basic extract showed interesting cytotoxicity in replicate tests. *C. majus*, Greater Celandine, a traditional medicinal herb widespread in Europe, represents a rich source of isoquinoline alkaloids and flavonoids [1], employed locally to remove warts, papillomas and condylomas [2]. Under the presumption that certain alkaloid constituents contained in *C. majus* exhibit inhibitory properties on cancer cell growth, our attention was focused on the toxicity of the basic extract. The natural compound coptisine was isolated from the basic extract and identified as the cytotoxic component of *C. majus* aerial parts. The alkaloid coptisine was then synthesised in gram amount from berberine.

Natural and synthetic coptisine were tested in an enlarged tumour cell line panel: carcinoma cell line LoVo and its subline resistant to doxorubicin (Dx), murine leukaemia cell line L1210 and its subline resistant to cisplatin (CDDP).

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## 2. Materials and methods

### 2.1. Extraction scheme

Plant material was extracted according to the Harborne procedure [3]. Briefly, the vegetable tissue was reduced into small particles of uniform size in a homogeniser: 35 g of fresh material was treated with 200 ml MeOH–$H_2O$ (2:1 v/v) at 4°C for 5 min. The hydroalcoholic extract was recovered by filtration. The vegetable material was extracted again with 100 ml of the above solution and filtered. The two hydroalcoholic extracts were mixed and reduced in volume (1/10) to obtain the crude extract (CE). The CE was then acidified with 2 N $H_2SO_4$ (pH 2) and then extracted in a separatory funnel with $CHCl_3$ (50 ml × 3). The mixed organic phases were treated with anhydrous sodium sulfate, filtered and reduced in volume (1/10). This step gave the moderately polar extract (MPE). The residual aqueous phase was alkalinised with 30% $NH_4OH$ (pH 9) and then extracted a first time with 50 ml of $CHCl_3$ and then with $CHCl_3$–MeOH (50 ml × 2). The three organic phases were mixed, treated with anhydrous sodium sulfate and reduced in volume (1/10). The organic extract was defined as the basic extract (BE).

The CE, MPE and BE extracts were first monitored by TLC ($SiO_2$ $F_{254}$, Merck, Darmstadt, Germany). The mobile phase was $CHCl_3$–MeOH–$H_2O$–$CH_3COOH$ (85:15:1:2 v/v).

The CE, MPE and BE extracts were subsequently monitored by HPLC under isocratic conditions. Column: Alltech silica gel (250 × 4 mm i.d., 10 μm particle size). The mobile phase was 0.015 M sodium acetate in acetic acid–methanol–1,4 dioxane–isopropanol (2:68:10:20 v/v). Detection was at 280 nm, with a flow rate of 1.5 ml/min [4].

### 2.2. Fractionation of the BE extract

The BE extract (270 mg) adsorbed on celite was fractionated on $SiO_2$ packed column. $SiO_2$ was imbibed with $CHCl_3$–MeOH–$H_2O$–$CH_3COOH$ conc. (90:10:1:1 v/v) with slow addition of methanol until the solvent solution was clear. We collected 60 fractions (8–10 ml each) before coptisine isolation.

Then the column chromatography mobile phase was changed in order to elute other more polar components. The new mobile phase $CHCl_3$–MeOH (50:50 v/v) allowed the collection of 10 fractions (8–10 ml) from 61 to 70. Eventually, the column chromatogram was washed with 100% methanol, thus obtaining 10 fractions from 71 to 80.

The TLC system showed that fractions 64–66 had a yellow–green spot as the main component; these fractions were then pooled together. The main component was isolated, crystallised as a chloride and identified as coptisine, on the basis of MS and IR data. MS and NMR spectra and biological activity of recovered coptisine were performed in comparison to berberine chloride (Sigma Chemical Co., UK), a protoberberine alkaloid structurally related to the coptisine.

### 2.3. Cytotoxicity assay procedure

The CE, MPE and BE crude extracts were first tested against *A. salina* Leach (brine shrimp) nauplii, and cultured human tumour cell, the colon carcinoma HT 29.

1. The bioassay against *A. salina* Leach, brine shrimp nauplii, was performed as described previously [5] and developed with minor modifications. Cysts were from Aquafauna Bio-Marine Inc., Hawthorne, CA. To obtain nauplii the dry cysts (ca 50 mg) were hatched in artificial sea water prepared from sea salt (Sigma) 40 g/l in double-distilled water, in a rectangular box (20 × 30 cm) in a warm room (25–27°C) in the darkness. After 48 h incubation, the nauplii were collected by pipette after attracting them with a light source and after separation from their shells. Plant extract cytotoxicity was evaluated in a range of 1000, 100 and 10 μg/ml, each test was made on five groups of 10 animals for each dose of toxin, with a final test mixture of 5 ml. The plant extracts were dissolved in DMSO (stock solution) prior to dilution. The maximum DMSO concentration was 0.5%. Negative controls using 0.5% DMSO were used. Each assay was performed on nauplii after 48 h. Food (yeast) was added during the hatching not during test procedure. Survival data were evaluated as 'immobility'. In the cases where control deaths occurred the data were corrected using Abbott's formula:% death = [(test − control)/control] × 100. $LD_{50}$ was determined from the 24 h counts using the probit analysis method.
2. Cultured human tumour cell (colon carcinoma HT 29 line) established cultures have been propagated in McCoy's 5a medium with 15% foetal bovine serum.

### 2.4. Enlarged cancer cell line panel

1. Human tumour colon carcinoma cell line LoVo and its subline resistant to Dx were cultured in Ham's F12 medium supplemented with 10% foetal calf serum + 1% l-glutamine (200 mM) + 1% antibiotic solution + 2% HEPES buffer.
2. Murine leukaemia cell line L1210 and its subline resistant to CDDP L1210/Pt were cultured in RPMI medium supplemented with 20% foetal calf serum + 1% l-glutamine (200 mM) + 0.5% β-mercaptoethanol.

All the cell lines were maintained at 37°C in an atmosphere of 5% $CO_2$ and 90% relative humidity.

### 2.5. MTT assay

The cytotoxic activity of the compounds was determined by the MTT colorimetric assay. The MTT colorimetric assay [6] is based on the mitochondrial reduction of tetrazolium salt by living cells. The viable cell number is proportional to the production of formazan salts. The crystals of formazan were dissolved in DMSO and the optical density was measured spectrophotometrically (Microplates reader, Series 750, Cambridge Technology Inc.).

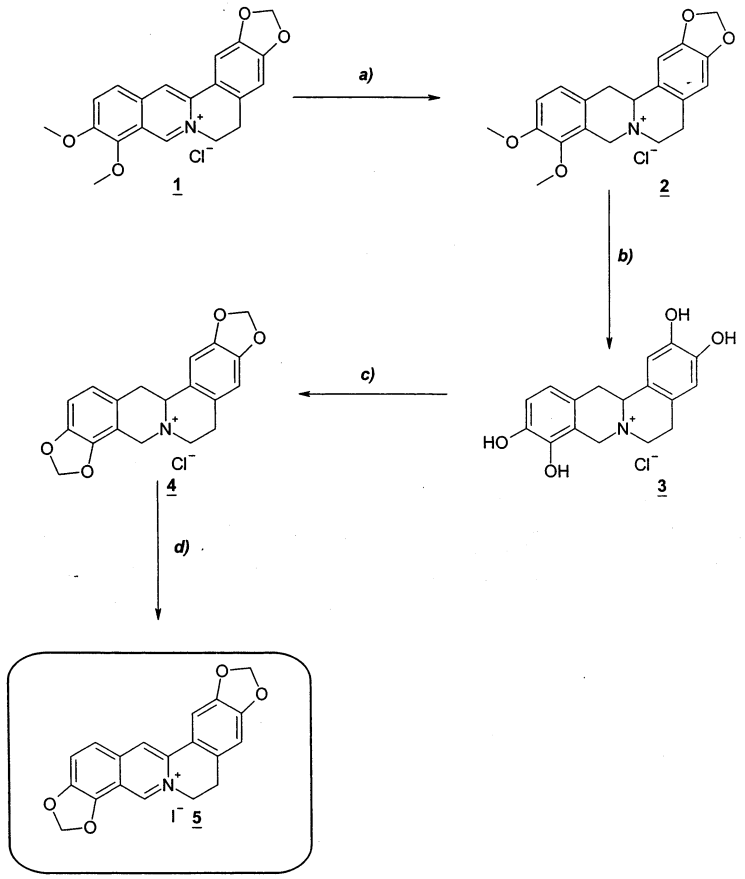
### 2.6. Experimental procedures in the synthesis of coptisine

#### 2.6.1. First step

For the preparation of tetrahydroberberine (**2**) berberine chloride (**1**) (10 g) suspended in 50% aq. acetic acid (150 ml) was hydrogenated in presence of Adam's catalyst (200 mg) in Parr hydrogenator at 3–4 atm. The absorption of 2 mol of hydrogen took 10–12 h. At the end of the reduction the solution was diluted with water (150 ml) and the catalyst filtered off. The filtrate was basified using aq. ammonia (100 ml) and the almost white tetrahydroberberine chloride was collected by filtration. Recrystallisation with hot ethanol gave tetrahydroberberine (**2**) as colourless crystals (7 g) in 85% yield. The TLC solvent system was hexane–ethyl acetate, 8:2, $R_f = 0.8$ (see Scheme 1).

#### 2.6.2. Second step

For the preparation of tetrahydroxytetrahydroberberine chloride (**3**) to a stirred solution of tetrahydroberberine (**2**) (3.7 g, 9.8 mmol) in dry DCM (120 ml) under nitrogen at −40°C, a solution of 1 M borontribromide in DCM (60 ml, 59 mmol) was added over a




a) 3-4 atm $H_2$, $PtO_2$, AcOH/$H_2O$ 1/1, 10-12 h; 85% yield

b) $BBr_3$ (6 eq.), $CH_2Cl_2$, - 40°C, then 20°C, 12 h; 96% yield

c) $CH_2BrCl$ (2 eq.), CsF (10 eq.), DMF, 95°C, 24 h; 20% yield

d) $I_2$ (2.25 eq.), AcONa (1.4 eq.), EtOH/AcOH 2/1, 50°C, 20', then 20°C, 12h; 67% yield

Scheme 1. Four-step procedure in the synthesis of coptisine.

Table 1
Chemical and physical data of the synthesised coptisine

| | | | |
|---|---|---|---|
| UV | $\lambda_{max}$ | $E^{1\%}_{1\,cm}$ | $\varepsilon$ |
| Solvent HCl 0.1 N in MeOH–$H_2O$ | 266 | 494 | −22 115 |
| | 357 | 506 | 22 630 |
| | 458 | 113 | 5039 |
| FT-IR | ($cm^{-1}$) | | |
| KBr pellets | 3037 | | |
| | 2899 | | |
| | 1508 | | |
| | 1478 | | |
| | 1215 | | |
| | 1057 | | |
| | 1039 | | |
| $^1$H NMR | (ppm) | | |
| Solvent: DMSO-$d_6$ | 9.954 | s | 1H |
| | 8.958 | s | 1H |
| | 8.032 | d | 1H |
| | 7.838 | s | 1H |
| | 7.793 | m | 1H |
| | 7.088 | s | 1H |
| | 6.541 | s | 2H |
| | 6.176 | s | 2H |
| | 4.875 | m | 2H |
| | 3.200 | m | 2H |
| Elemental analysis | % Theor. | % Found | |
| C | 51.03 | 50.35 | |
| H | 3.16 | 3.21 | |
| N | 3.13 | 3.07 | |
| I | 28.38 | 27.50 | |
| HPLC | r.t.: 13.8° | | |

Conditions — Column: TOSOHAAS ODS; 25 cm × 4.6 mm; p.s.: 5 μm; Eluent: water–acetonitrile–dioxan, 65:30:5 with 20 mM $KH_2PO_4$ + 5 mM octansulfonic acid sodium salt; pH 2.7 ($H_3PO_4$). Flow eluent: 1 ml/min. Detector UV at 240 nm
m.p. (DSC) > 270°C

period of 20 min so that a constant temperature of −40°C was maintained throughout the addition. The reaction was allowed to come to room temperature (r.t.) and stirred for a further 12 h. After cooling the reaction mixture to 0°C the excess of borontribromide was quenched by slow addition of methanol (50 ml). The reaction mixture was concentrated to half its volume and the insoluble tetrahydroxy compound (**3**) was removed by filtration and washed with DCM (20 ml). The crude tetrahydroxytetrahydroberberine (**3**) (3.2 g, 96%) was used as such in the next step without any further purification.

### 2.6.3. Third step

A solution of **3** (7.05 g, 19 mmol) in dry DMF (100 ml) under nitrogen was heated to 60°C and a solution of bromochloromethane (2.5 ml, 39 mmol) in DMF (5 ml) was added over a period of 10 min. The temperature of the reaction was raised to 95°C and left for 24 h. After cooling to r.t., the inorganics were filtered off and the filtrate was concentrated at reduced pressure. The dark-coloured residue was diluted with water (50 ml) and extracted with ether (3 × 100 ml). The combined organic layers were washed with water, dried over anhydrous $MgSO_4$, filtered and concentrated to furnish a yellow solid. Recrystallisation with ethyl acetate gave the tetrahydrocoptisine (**4**) (1.5 g, 20%). The TLC solvent system was hexane–ethyl acetate, 8:2, $R_f = 0.6$.

### 2.6.4. Fourth step

For the preparation of coptisine iodide (**5**) a mixture of tetrahydrocoptisine (**4**) (0.65 g, 1.8 mmol) and sodium acetate (2.08 g, 2.5 mmol) was dissolved in a 2:1 mixture of boiling ethanol and acetic acid (30 ml); into this warm solution at 50°C a solution of iodine (1.04 g, 4.05 mmol) in ethanol (20 ml) was added slowly over a period of 20 min. The mixture was stirred overnight at r.t. and the precipitated coptisine iodide (**5**) was removed by filtration and washed with ethanol. It was further purified by heating in aq. acetone to give coptisine iodide (**5**) as yellow crystal (0.55 g, 67%) decomposing at 285°C.

Chemical and physical data of synthetic coptisine are summarised in Table 1.

## 3. Results

Plant crude extracts obtained from plant material following a standard procedure [3] were tested in a preliminary step using a rapid cytotoxic-like assay: *A. salina* Leach, brine shrimp nauplii, and the antiproliferative test against HT 29 human colon carcinoma cultured cells. The biological activity of the methanolic extract of papaveraceous *C. majus* was significantly interesting against brine shrimp (*A. salina* Leach) nauplii and colon carcinoma HT 29 cells. Fractionation of the crude extract and bioassay-guided procedures showed that the cytotoxic activity was concentrated in the basic extract $LD_{50} = 250$ μg/ml in *A. salina* and $IC_{50} = 1.14$ μg/ml in colon carcinoma HT 29 cell proliferation.

Solvent partitioning of the basic extract concentrated the cancer cell growth inhibitory activity into a methanol-soluble fraction that was further separated by column chromatography (see Section 2). The chromatographic separation over a large silica gel column of the BE yielded an active fraction in which the cytotoxicity was concentrated: $LD_{50}$ 98 μg/ml (*A. salina*) and $IC_{50}$ 0.49 μg/ml (HT 29 cell line). After purification and crystallisation of the active fraction, yellow fine needles of an alkaloid compound were obtained. The compound was identified as coptisine (**1**) on the basis of TLC, IR, MS and $^1$H NMR data, by comparison with berberine (**2**), a protoberberine alkaloid structurally related to coptisine (Fig. 1).

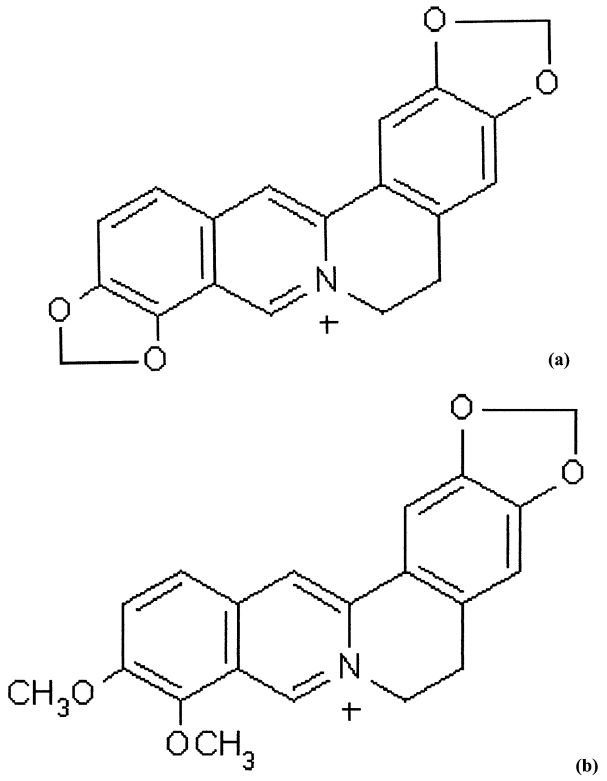



Fig. 1. (a) Coptisine chemical structure; and (b) berberine chemical structure.

The isolated coptisine was tested against an enlarged panel of cultured human cancer cells carcinoma cell line LoVo and its subline resistant to Dx and against murine leukaemia cell line L1210 and its subline resistant to CDDP. The results of activities were summarised in Fig. 2. Coptisine showed a similar cytotoxicity against the LoVo cell line ($IC_{50} = 0.87$ μg/ml) and L1210 ($IC_{50} = 0.87$ μg/ml), its cytotoxicity nevertheless was twice higher on HT 29 cell line ($IC_{50} = 0.49$ μg/ml). The alkaloid berberine, tested in comparison with coptisine, was more powerful on the leukaemic line ($IC_{50} = 0.33$ μg/ml) while it had a lower activity on HT 29 and LoVo cells ($IC_{50} = 1.89$ and 1.90 μg/ml, respectively). As regards the crossresistance with CDDP on L12010/CDDP cells or with Dx on LoVo/Dx cells, the isolated coptisine is partially crossresistant.

To confirm the identification of the activity and provide more material for biological testing the synthesis seemed more practical than re-isolation. The alkaloid coptisine was then synthesised from commercial berberine. To our knowledge, there are no early reports on coptisine synthesis. The only mention is in a footnote of a Japanese paper where the authors stated that the conversion of berberine to coptisine will be possible and that it would be published in a separate paper, which has never appeared [7].

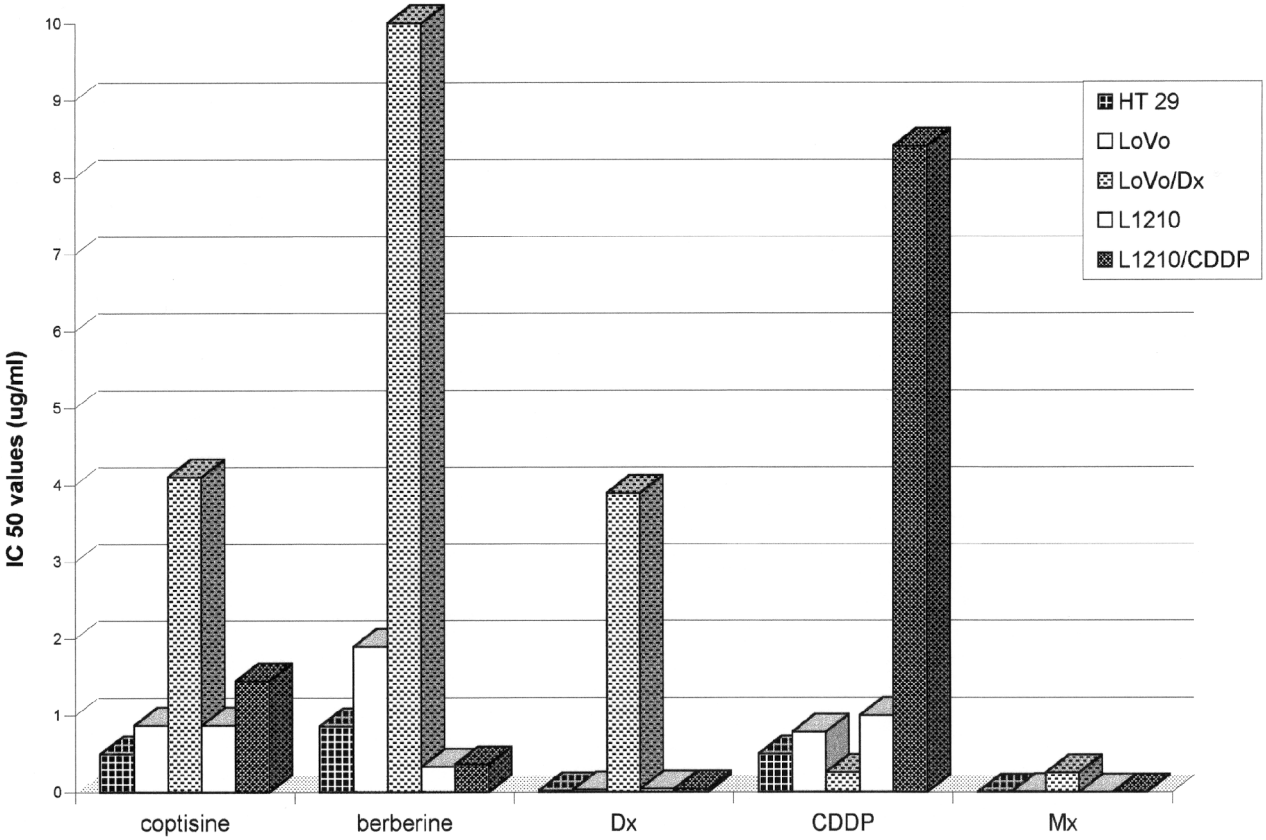



Fig. 2. $IC_{50}$ values (μg/ml) of natural coptisine and berberine evaluated on HT 29, LoVo and LoVo/Dx, L1210 and L1210/CDDP tumour cell lines.

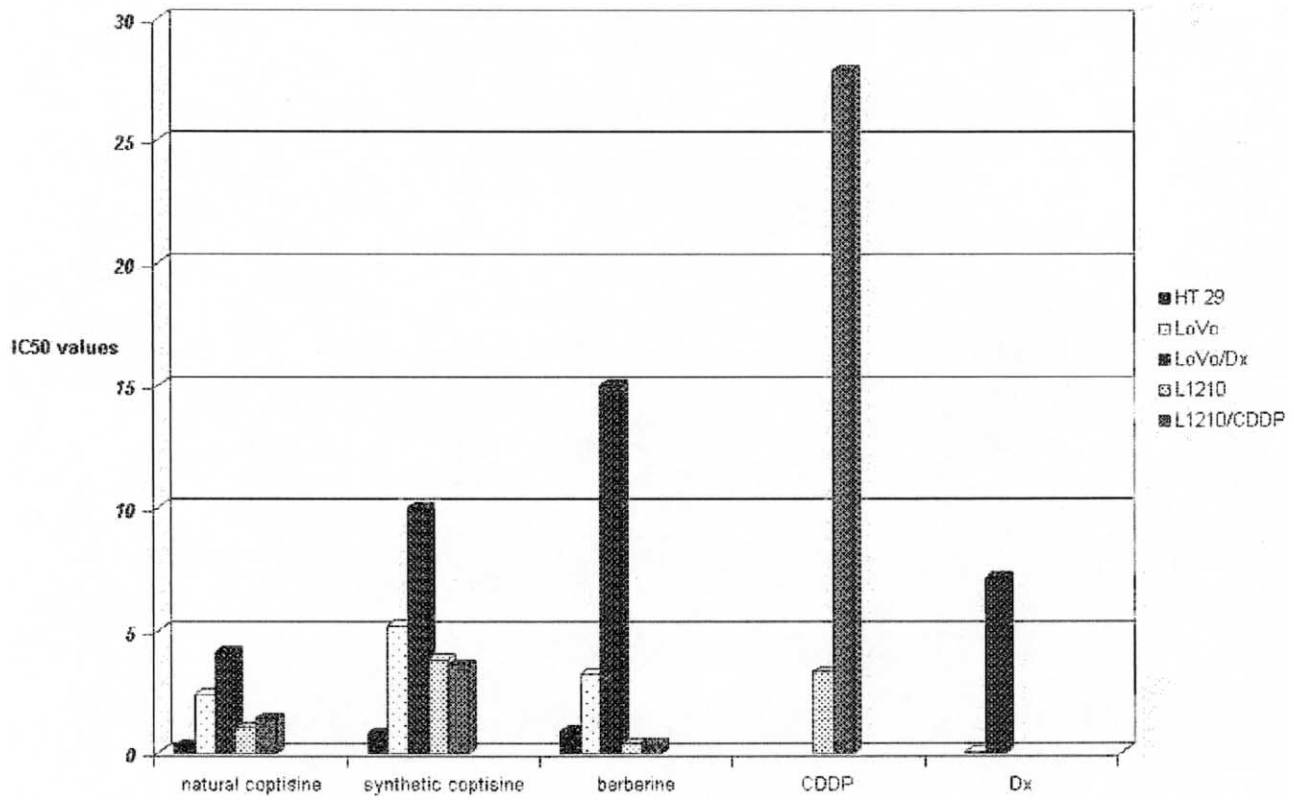



Fig. 3. $IC_{50}$ values (μg/ml) of natural coptisine, synthetic coptisine and berberine evaluated on HT 29, LoVo and LoVo/Dx, L1210 and L1210/CDDP tumour cell lines.

The synthesis of coptisine was achieved in four steps starting from berberine chloride (**1**). Hydrogenation of **1** in presence of Adam's catalyst gave the tetrahydroberberine chloride (**2**) in 85% yield. The conversion of **2** to the tetrahydroxy derivative (**3**) was brought about using boron tribromide in 96% yield. An attempt to bis methylate **3** to obtain the tetrahydrocoptisine (**4**) under PTC conditions using adogen as catalyst was futile. The bismethylation was accomplished in 20% yield using caesium fluoride and bromochloromethane in DMF. The oxidation of **4** using iodine in acetic acid gave the desired target molecule coptisine iodide (**5**) in 67% yield. Chemical and physical data of the synthetic material matched those of the product isolated from the plant, as reported in Scheme 1.

The cytotoxicity of natural coptisine and synthetic coptisine iodide was evaluated:

- versus berberine and Dx on human colon carcinoma cell line LoVo and its subline resistant to Dx, LoVo/Dx;
- versus berberine and CDDP on murine leukaemia cell line L1210 and its subline resistant to CDDP, L1210/Pt.

On the basis of all the developed tests, natural and synthetic coptisines showed a similar cytotoxicity trend (Fig. 3): coptisine is very toxic on HT 29 cells (natural $IC_{50} = 0.66$ μg/ml and synthetic $IC_{50} = 0.88$ μg/ml). Coptisine had an antiproliferative activity on LoVo cells: natural $IC_{50} = 2.38$ μg/ml and synthetic $IC_{50} = 5.2$ μg/ml. On L1210 cell line berberine is more toxic than natural and synthetic coptisine: $IC_{50} = 0.37$ μg/ml for berberine and $IC_{50} = 1.02$ μg/ml for natural coptisine and $IC_{50} = 3.82$ μg/ml for synthetic coptisine. In all the considered tests it is seen that natural coptisine seemed more active than the synthetic one. Perhaps the natural coptisine, recrystallised as chloride, was more soluble in water than the synthesised iodide salt.

The cytotoxicity results of natural and synthetic coptisine are summarised below

LoVo Mitox > Dx > CDDP > coptisine > berberine
HT 29 Mitox > Dx > CDDP = coptisine > berberine
L1210 Mitox > Dx > berberine > coptisine ≥ CDDP

In conclusion we can affirm that the alkaloid coptisine has been identified as the most significant cancer cell growth inhibitory substance from the aerial parts of *C. majus* and should deserve further studies to assess its antitumour efficacy.

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