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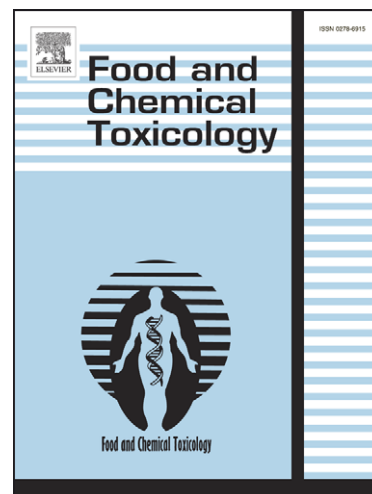
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A novel toxic alkaloid from poison hemlock (*Conium maculatum* L., Apiaceae):  
identification, synthesis and antinociceptive activity

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## Abstract

2-Pentylpiperidine, named conmaculatin, a novel volatile alkaloid related to coniine was identified from the renowned toxic weed *Conium maculatum* L. (Apiaceae). The structure of conmaculatin was corroborated by synthesis (8 steps starting from cyclohexanol, overall yield 12%). Conmaculatin's strong peripheral and central antinociceptive activity in mice was observed in a narrow dose range (10-20 mg/kg). It was found to be lethal in doses higher than 20 mg/kg.

*Keywords:* *Conium maculatum* L., 2-pentylpiperidine, volatile alkaloid, synthesis, antinociceptive activity, toxicity

## 1. Introduction

*Conium maculatum* L., poison hemlock, is one of the highly poisonous perennial herbaceous flowering plants of the family Apiaceae, native in temperate regions of Europe, West Asia, as well as North Africa, but has been introduced and naturalised in many other areas, including Asia, North America, Australia, and New Zealand (Schep et al., 2009). Upon drying the toxicity of the plant material is greatly reduced, although not gone completely (Lopez et al., 1999), implying that the toxic principle might be a volatile one or unstable. Up to now ten volatile alkaloids have been identified (Lang and Smith, 1998; Reynolds, 2005) of which coniine and  $\gamma$ -coniceine are generally the most abundant ones and they account for most of the plant's acute and chronic toxicity (Lopez et al., 1999). The latter, which is the first formed biosynthetically and is more toxic, often accompanies coniine, sometimes occurring in greater quantities (Reynolds, 2005). General symptoms of hemlock poisoning include an initial

stimulation of motor nerve endings and central nervous system, followed by paralysis and depression, respectively, problems in movement, slow and weak, then later, rapid pulse, hyperventilation, urination, and finally coma and death (Vetter, 2004).

Qualitative structure - activity (chronic toxicity, teratogenic effects) relationship study of *C. maculatum* alkaloids showed that the side chain of the piperidine moiety must be at least a propyl group to have any effect (Lopez et al., 1999), and that it has to be in the position 2 (Bunch et al., 1992). Recently, the analgesic effect of coniine has been studied in thermal and chemical pain models as well, and the results of both tests indicated an antinociceptive effect of coniine. It was also reported that the antinociceptive effect of morphine was potentialized by coniine, whose action was in turn inhibited by the nicotinic receptor blocker mecamylamine (Arihan et al., 2009). The medicinal importance of hemlock (e.g. unripe *C. maculatum* seeds were dried and stored to be used as an antispasmodic, a sedative or an analgesic (Bowman and Snaghvi, 1963)) is very limited because of the closeness between therapeutic and poisonous levels (Holm, 1997).

Previous studies showed that all tissues of *C. maculatum* are very rich in alkaloids (Corsi and Biasci, 1998; fruits being the richest with up to 1% (w/w) per alkaloid (Lopez et al., 1999)), but the amount and mutual ratio of the several different alkaloids depends on plant variety, ecological conditions and the stage of phenological development (Vetter, 2004). However, most of the structural elucidation (now regarded as classical and perhaps obsolete) work done on these alkaloids was in the 1950s, through 1960s and ending in the 1970s with the concluding biosynthetic studies (Reynolds, 2005). A more recent reexamination of this plant species (Lang and Smith, 1998) revealed that it still presents a source of new naturally occurring compounds (2-pentyl-3,4,5,6-tetrahydropyridine and 5-hydroxy-2-pentylpiperidine). With these results as our motivation, and the belief that modern instrumental analytical tools unavailable in the 1970s would provide means for the

identification of even minor metabolites, and in continuation of our previous studies on the volatile (Radulović et al., 2008) and non-volatile (Radulović and Đorđević, 2011) chemistry of *C. maculatum*, the goal of this work was set to perform a detailed analysis of the volatile alkaloid content of poison hemlock from Serbia in hope of finding new compounds with potentially interesting biological and pharmacological activities.

## 2. Material and methods

### 2.1. General experimental procedures

All chemicals were commercially available and used as received (Aldrich, USA; Merck, Germany; Fluka, Germany), except that the solvents were purified by distillation. Preparative medium-pressure liquid chromatography (MPLC) was performed with a pump module C-601 and a pump controller C-610 Work-21 pump (Büchi, Switzerland) and was carried out on pre-packed column cartridges (40 x 75 mm, Silica-gel 60, particle size distribution 40-63  $\mu\text{m}$ , Büchi), whereas precoated Al silica gel plates (Merck, Kieselgel 60 F<sub>254</sub>, 0.2 mm) were used for analytical TLC analyses. The spots on TLC were visualized by UV light (254 nm) and by spraying with 50% (v/v) aq. H<sub>2</sub>SO<sub>4</sub> followed by heating. The GC-MS analyses were performed on a Hewlett-Packard 6890N gas chromatograph equipped with fused silica capillary column DB-5MS (5% phenylmethylsiloxane, 30 m  $\times$  0.25 mm, film thickness 0.25  $\mu\text{m}$ , Agilent Technologies, USA) and coupled with a 5975B mass selective detector from the same company as detailed in section 2.6. The IR measurements (ATR-attenuated total reflectance) were carried out using a Thermo Nicolet model 6700 FTIR instrument (Waltham, USA). The <sup>1</sup>H and <sup>13</sup>C NMR spectra have been recorded on a Varian Gemini 200 spectrometer operating at 200 and 50 MHz, respectively. 2D experiments (<sup>1</sup>H - <sup>1</sup>H COSY and

HETCOR) were run on the same instrument with the usual pulse sequences. All NMR spectra were measured at 25 °C in CDCl<sub>3</sub> with tetramethylsilane (TMS) as internal standard. Chemical shifts are reported in ppm ( $\delta$ ) and referenced to TMS ( $\delta_{\text{H}} = 0$  ppm) in <sup>1</sup>H NMR spectra, or to residual CHCl<sub>3</sub> ( $\delta_{\text{H}} = 7.25$  ppm) and <sup>13</sup>CDCl<sub>3</sub> ( $\delta_{\text{C}} = 77$  ppm) in heteronuclear 2D spectra. Scalar couplings are reported in hertz (Hz).

### *2.2. Plant material*

Plant material was collected in the blooming and fruit forming stages during the spring and summer of 2009 from three different locations in the area of the city of Niš, southeastern Serbia (three or four collection per location). One of the authors (N.R.) did the botanical identification of the plant material during collection. The identity of the voucher specimens (No. from 200905 to 200916) was corroborated by a botany professor/curator from the Herbarium of the Department of Biology, Faculty of Science and Mathematics, Niš.

The gathered plant material was transported from the field to the laboratory within 30 min after collection and subjected to extraction as detailed bellow without delay.

### *2.3. Test animals*

All experiments were performed with male Swiss mice (18-25 g) obtained from our own animal facilities (Laboratório de Farmacologia da Inflamação e do Óxido Nítrico). Animals were maintained in a room with controlled temperature  $22 \pm 2$  °C for 12 h light/dark cycle with free access to food and water. Twelve hours before each experiment animals received only water, in order to avoid food interference with substances absorption. Animal care and research protocols were in accordance with the principles and guidelines adopted by the

Brazilian College of Animal Experimentation (COBEA), approved by the Biomedical Science Institute/UFRJ, Ethical Committee for Animal Research, and received the number DFBCICB-015.

#### *2.4. Isolation of the essential oils and solvent extracts*

Roots and aerial parts of *C. maculatum* were analyzed separately in the florescence stage whereas the analyzed plant material collected during the fruit forming stage consisted either of fruits or roots alone. Aerial parts, as well as fruits, were submitted to both hydrodistillation and alkaline solvent extraction, while the roots were submitted only to solvent extraction.

##### *2.4.1. Hydrodistillation*

Fresh plant material (leaves and flowers, or fruits) was subjected to hydrodistillation with *ca.* 2.5 L of distilled water for 2.5 h using the original Clevenger-type apparatus to produce the yellowish mousy deterrent in smell essential oil. The obtained oils were separated by extraction with diethyl ether (Merck, Germany), dried over anhydrous MgSO<sub>4</sub> (Aldrich, USA) and stored at -18 °C until analysis.

##### *2.4.2. Alkaline solvent extraction*

Fresh plant material (leaves and flowers, or fruits) was appropriately cut into small pieces and macerated with a two phase system consisting of equal volumes of aq. solution of KOH (20% w/w) and diethyl ether (Et<sub>2</sub>O). After seven days with occasionally mixing the plant material

was filtered off, ethereal layer was separated from the water layer, washed with H<sub>2</sub>O, dried over anhydrous MgSO<sub>4</sub> and concentrated *in vacuo*.

#### 2.4.3. Roots extraction

Fresh roots were chopped into small pieces and extracted with Et<sub>2</sub>O at room temperature for seven days. Then the ethereal layer was separated from the plant material, dried over anhydrous MgSO<sub>4</sub> and concentrated under reduced pressure.

#### 2.5. Synthesis of conmaculatin

Ethyl 2-oxocyclopentanecarboxylate (**4**) was synthesized starting from cyclohexanol (**2**) in a classic three reaction sequence (Vogel, 1989) with a total yield of 46%. 1-Iodopentane (**6**) was obtained in 67% yield from 1-pentanol (**5**) following a standard procedure (Vogel, 1989).

##### 2.5.1. Ethyl 2-oxo-1-pentylcyclopentanecarboxylate (**7**)

Anhydrous K<sub>2</sub>CO<sub>3</sub> (107 g, 0.750 mol) and 240 mL of dry acetone were mixed, then with stirring, to the resulting mixture a soln. of **4** (0.190 mol) in 125 mL of dry acetone was dropwise added. After stirring at room temperature for 35 min, 38 mL (0.287 mol) of **6** was added dropwise too. The reaction mixture was refluxed for 2 additional hours and then left overnight. The solvent was evaporated *in vacuo* and the remaining slurry was extracted several times with Et<sub>2</sub>O. The combined extracts were washed with H<sub>2</sub>O, dried over anhydrous MgSO<sub>4</sub> and concentrated under reduced pressure to give 39.9 g of the crude product (consisting of *ca.* 93% of the wanted product **7** and 7% of its regioisomer **7a**, ethyl 2-oxo-3-



pentylcyclopentanecarboxylate, as inferred from a GC-MS analysis). The total yield of both regioisomers was 93%.

Ethyl 2-oxo-1-pentylcyclopentanecarboxylate (**7**);  $R_t$  18.15 min, RI (DB-5MS) 1561; EIMS, 70 eV,  $m/z$  (rel. int.): 226 (0.2)  $[M]^+$ , 198 (0.6), 181 (8.2), 169 (4.9), 156 (100.0)  $[M - C_5H_{10}]^+$ , 141 (10.5), 128 (11.9), 110 (48)  $[M - C_5H_{10} - EtOH]^+$ , 95 (15.9), 81 (10.2), 67 (17.0), 55 (26.9), 41 (22.2); Ethyl 2-oxo-3-pentylcyclopentanecarboxylate (**7a**);  $R_t$  20.33 min, RI DB-5MS 1658; EIMS, 70 eV,  $m/z$  (rel. int.): 226 (7.6)  $[M]^+$ , 197 (0.3), 181 (7.5), 169 (1.7), 156 (25.6)  $[M - C_5H_{10}]^+$ , 141 (0.9), 128 (13.3), 110 (100.0)  $[M - C_5H_{10} - EtOH]^+$ , 99 (0.9), 82 (9.5), 68 (3.4), 55 (18.7), 43 (13.7).

### 2.5.2. 2-Pentylcyclopentanone (**8**)

A slightly modified procedure of Ikan and Ravid (1974) was applied. The mixture obtained after alkylation step (29.4g, consisting of *ca.* 0.13 mol of **7** and **7a** according to GC-MS) was refluxed for 24 h in the presence of 100 mL of glacial acetic acid and 180 mL of 20% (w/w) HCl. The reaction mixture was then cooled, made alkaline with 40% (w/w) aqueous (aq.) solution of NaOH and extracted with Et<sub>2</sub>O. The organic layers were combined, washed with H<sub>2</sub>O, dried over anhydrous MgSO<sub>4</sub> and concentrated *in vacuo* to yield 13.8 g of reasonably pure **8** (according to GC-MS). The yield was 69%.

2-Pentylcyclopentanone (**8**);  $R_t$  10.35 min, RI (DB-5MS) 1242; EIMS, 70 eV,  $m/z$  (rel. int.): 154 (8.8)  $[M]^+$ , 125 (1.2), 112 (1.9), 97 (15.6), 91 (0.4), 84 (100.0)  $[M - C_5H_{10}]^+$ , 79 (2.0), 69 (7.0), 63 (0.2), 55 (18.6), 50 (0.3), 41 (20.4).

### 2.5.3. 2-Pentylcyclopentanone oxime (**9**)

2-Pentylcyclopentanone oxime was synthesized by the following modified general method for solvent free synthesis of oximes by Vukićević et al. (2006).  $\text{NH}_2\text{OH}\cdot\text{HCl}$  (12.8 g, 0.19 mol) was finely ground in a mortar with a pestle. Then NaOH and the ketone **8** were added in small portions in such a manner that new amounts were added only after appropriate homogenization. In total 7.4 g (0.19 mol) NaOH and 13.2 g of **8** (0.086 mol) was added. After standing on room temperature for 72 h, the reaction mixture was extracted three times with  $\text{Et}_2\text{O}$ . Combined ether extracts, dried over anhydrous  $\text{MgSO}_4$ , were concentrated under reduced pressure. The obtained crude product (14.3 g, consisting of *ca.* 92.5% of (*E*) - 2-pentylcyclopentanone oxime (**9**), 7% of (*Z*) - 2-pentylcyclopentanone oxime (**9a**) and 0.5% of ethyl 2-(hydroxyimino)-1-pentylcyclopentanecarboxylate (**9b**) as inferred from a GC-MS analysis) was a yellow oil. The yield was 98%.

(*E*) - 2-Pentylcyclopentanone oxime (**9**): Yellowish oil;  $R_t$  14.89 min, RI (DB-5MS) 1425; FTIR-ATR (neat)  $\text{cm}^{-1}$ : 3258.9 (O-H), 2926.5, 1670.7 (C=N), 1452.5, 1197, 919.6, 725.3;  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ ):  $\delta$  0.84 (3H, *t*, C5 -H), 1.16-1.42 (8H, *m*), 1.46-2.04 (4H, *m*), 2.26-2.66 (3H, *m*, C2-H and C5-H), 9.30 (1H, *br s*, C1=N-OH);  $^{13}\text{C}$  NMR (50 MHz,  $\text{CDCl}_3$ ):  $\delta$  13.9 (C-5'), 22.4, 22.5, 27.1, 27.2, 31.6, 31.7, 32.1 (C-5), 42.9 (C-2), 169.3 (C-1); EIMS, 70 eV,  $m/z$  (rel. int.): 169 (1)  $[\text{M}]^+$ , 152 (2.1), 140 (1.9), 122 (1.3), 112 (19.7), 99 (100.0)  $[\text{M} - \text{C}_5\text{H}_{10}]^+$ , 91 (0.7), 82 (5.7), 74 (0.1), 67 (19.6), 55 (8.6), 41 (15.0); (*Z*) - 2-Pentylcyclopentanone oxime (**9a**):  $R_t$  14.58 min, RI (DB-5MS) 1412; EIMS, 70 eV,  $m/z$  (rel. int.): 169 (1.3)  $[\text{M}]^+$ , 152 (7.7), 140 (2), 122 (4.3), 112 (24.2), 99 (100.0)  $[\text{M} - \text{C}_5\text{H}_{10}]^+$ , 91 (1.1), 82 (15.4), 67 (21.8), 55 (14.2), 41 (23.6); Ethyl 2-(hydroxyimino)-1-pentylcyclopentanecarboxylate (**9b**):  $R_t$  22.36 min, RI (DB-5MS) 1751; EIMS, 70 eV,  $m/z$  (rel. int.): 195 (5.2), 171 (36.5)  $[\text{M} - \text{C}_5\text{H}_{10}]^+$ , 155 (8.2), 135 (6.1), 125 (100.0)  $[\text{M} - \text{C}_5\text{H}_{10} - \text{EtOH}]^+$ , 112 (11.2), 96 (10.4), 80 (13.2), 67 (16.2), 55 (15.6), 41 (24.2).

#### 2.5.4. 6-Pentylpiperidin-2-one (**10**)

The Beckmann rearrangement was achieved by adapting the procedure developed by Hildebrand and Bogert (1936) for 6-methylpiperidine-2-one. A solution of 8.85 g of **9** and 8.9 mL aq. 75% (w/w) H<sub>2</sub>SO<sub>4</sub> was placed in a dropping funnel at the top of the flask containing 3 mL of H<sub>2</sub>SO<sub>4</sub> of the same concentration. The flask was heated with stirring to approximately 108 °C and then the oxime solution was run in very slowly. When all had been added, the flask was allowed to cool to room temperature. The reaction mixture was rinsed into a beaker with an equal volume of H<sub>2</sub>O, then 1.2 g of charcoal was added and the water layer was decanted from the oil, made alkaline with a NaOH (40-50%, w/w) aq. solution and extracted with CHCl<sub>3</sub>. The chloroform extracts were collected, dried over anhydrous MgSO<sub>4</sub> and concentrated *in vacuo* to give 3.41 g of the Beckmann rearrangement and fragmentation products mixture (consisting of *ca.* 76% of wanted product **10**, 18% of 3-pentylpiperidin-2-one (**10a**), 1% of 5-hexyldihydrofuran-2(3*H*)-one (**10b**) and a few more minor products inferred from a GC-MS analysis). The mentioned oil was worked up in the same manner to give 4.26 g of the crude product (consisting of *ca.* 57% of **10**, 27% of **10a**, 7% of **10b**, 2% of 6-pentyltetrahydro-2*H*-pyran-2-one (**10c**) etc.). The overall yield of the Beckmann rearrangement products, **10** and **10a**, was 77%, whereas the yield of **10** was 57%. The mixture obtained by extraction of the water layer was fractioned by MPLC using a gradient of hexane and Et<sub>2</sub>O to give 0.55 g of pure **10**. Additionally, fraction 8, Et<sub>2</sub>O-*n*-hexane (4:1), proved to be (and a surprise to us) the crystalline (*E*) - 2-pentylcyclopent-2-enone oxime (**10d**, 70 mg).

6-Pentylpiperidin-2-one (**10**); Colorless crystalline; R<sub>t</sub> 19.69 min, RI (DB-5MS) 1629; FTIR-ATR (neat) cm<sup>-1</sup>: 3203.1 (N-H), 2927.2, 2857.8, 1657.9 (C=O), 1406.8, 1166.1, 957.6, 726.6; <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>): δ 0.82 (3H, *t*, C5 -H), 1.12-1.46 (9H, *m*), 1.50-1.72 (1H,

*m*), 1.74-1.92 (2H, *m*), 2.10-2.40 (2H, *m*, C2-H), 3.35 (1H, *sextet*, C5-H), 6.48 (1H, *s*, N-H);  $^{13}\text{C}$  NMR (50 MHz,  $\text{CDCl}_3$ ):  $\delta$  13.8 (C-5'), 19.6, 22.3, 24.7, 28.2, 31.1, 31.5, 36.7 (C-3), 53.0 (C-6), 172.7 (C-2); EIMS, 70 eV, *m/z* (rel. int.): 169 (2.8)  $[\text{M}]^+$ , 141 (0.1), 124 (0.1), 112 (0.5), 98 (100.0)  $[\text{M} - \text{C}_5\text{H}_{11}]^+$ , 93 (0.1), 82 (0.7), 77 (0.1), 70 (5.1), 65 (0.2), 55 (23.1), 41 (5.1); 3-Pentylpiperidin-2-one (**10a**);  $R_t$  19.11 min, RI (DB-5MS) 1603; EIMS, 70 eV, *m/z* (rel. int.): 169 (1.2)  $[\text{M}]^+$ , 154 (0.4), 140 (1.0), 126 (6.1), 112 (25.4), 99 (100.0)  $[\text{M} - \text{C}_5\text{H}_{10}]^+$ , 94 (0.4), 84 (8.2), 79 (0.7), 69 (4.4), 55 (14.3), 41 (12.9); 5-Hexyldihydrofuran-2(3*H*)-one (**10b**);  $R_t$  15.97 min, RI (DB-5MS) 1469; EIMS, 70 eV, *m/z* (rel. int.): 152 (0.2)  $[\text{M}]^+$ , 141 (0.4), 128 (8.2), 113 (2.3), 100 (4.3), 85 (100.0)  $[\text{M} - \text{C}_6\text{H}_{13}]^+$ , 69 (3.4), 55 (10.0), 41 (10.1); 6-Pentyltetrahydro-2*H*-pyran-2-one (**10c**);  $R_t$  16.65 min, RI (DB-5MS) 1497; EIMS, 70 eV, *m/z* (rel. int.): 152 (2.5)  $[\text{M}]^+$ , 141 (1.0), 134 (2.0), 123 (1.2), 114 (10.4), 108 (2.3), 99 (100.0)  $[\text{M} - \text{C}_5\text{H}_{11}]^+$ , 84 (4.9), 71 (40.8), 55 (31.3), 42 (29.5); (*E*) - 2-Pentylcyclopent-2-enone oxime (**10d**); Crystalline;  $R_t$  15.89 min, RI (DB-5MS) 1466; FTIR-ATR (neat)  $\text{cm}^{-1}$ : 3211.5 (O-H), 3060.9 (C=C-H), 2918.1, 1664.2 (C=N), 1463.5, 974.4, 723.5;  $^1\text{H}$  NMR (200 MHz,  $\text{CDCl}_3$ ):  $\delta$  0.87 (3H, *t*, C5 -H), 1.24-1.34 (4H, *m*, C3 -H and C4 -H), 1.52 (2H, *quintet*, C2 -H), 2.19 (2H, *tq*, C1 -H), 2.45 (2H, *m*), 2.71 (2H, *m*), 6.24 (1H, *m*, C3-H), 9.05 (1H, *br s*, C1=N-OH);  $^{13}\text{C}$  NMR (50 MHz,  $\text{CDCl}_3$ ):  $\delta$  13.9 (C-5'), 22.4, 25.4, 26.2, 27.1, 28.9, 31.7 (C-5), 140.6 (C-3), 141.6 (C-2), 167.8 (C-1); EIMS, 70 eV, *m/z* (rel. int.): 167 (30.4)  $[\text{M}]^+$ , 150 (26.0), 124 (26.1), 111 (100.0)  $[\text{M}^+ - \text{C}_4\text{H}_8]^+$ , 106 (15.9), 94 (44.9), 79 (35.4), 67 (21.1), 53 (19.4), 41 (30.3).

#### 2.5.5. 2-Pentylpiperidine (**1**)

A modified procedure of Davies and co-workers (2004) was used. Compound **10** (0.2 g) was dissolved in 30 mL of dry  $\text{Et}_2\text{O}$ . Powdered  $\text{LiAlH}_4$  (0.23 g) was added in small portions to the

stirred solution. The reaction mixture was refluxed for 16 h before being quenched with 2 M aq. KOH solution. After the ethereal layer was decanted, the resulting solid phase was extensively washed with CH<sub>2</sub>Cl<sub>2</sub>. The organic layers were combined, dried over anhydrous MgSO<sub>4</sub> and concentrated *in vacuo* to give 0.145 g of the crude product (consisting of *ca.* 95% of the wanted product **1** and 5% of the starting lactam **10**, as inferred from a GC-MS analysis) with characteristic amine odor. The yield was 75%. The crude product was dissolved in 5% aq. (w/w) HCl to convert **1** to the hydrochloride and the neutral organic impurities were extracted by Et<sub>2</sub>O. After neutralization of the water layer with 5% aq. (w/w) NaOH and extraction with Et<sub>2</sub>O, the combined extracts were dried over anhydrous MgSO<sub>4</sub> and concentrated under reduced pressure to yield the pure compound **1**. 3-Pentylpiperidine (**1a**) was obtained in an analogous way by reducing the lactam **10a**. 2-Pentylpiperidine (**1**); Colorless liquid; R<sub>t</sub> 9.36 min, RI (DB-5MS) 1205; FTIR-ATR (neat) cm<sup>-1</sup>: 3229.8 (N-H), 2923.2, 2853.0, 1451.9, 1328.0, 1118.6, 734.6; For <sup>1</sup>H and <sup>13</sup>C NMR spectra, see Table 1; EIMS, 70 eV, *m/z* (rel. int.): 155 (0.7) [M]<sup>+</sup>, 154 (1.4) [M - H]<sup>+</sup>, 140 (0.1), 126 (0.6), 112 (1.0), 96 (1.1), 84 (100.0) [M - C<sub>5</sub>H<sub>11</sub>]<sup>+</sup>, 77 (0.2), 70 (1.8), 56 (9.3) [M - C<sub>5</sub>H<sub>11</sub> - C<sub>2</sub>H<sub>4</sub>]<sup>+</sup>, 41 (5.0); 3-Pentylpiperidine (**10a**); R<sub>t</sub> 9.63 min, RI (DB-5MS) 1216; EIMS, 70 eV, *m/z* (rel. int.): 155 (1.2) [M]<sup>+</sup>, 154 (1.4) [M - H]<sup>+</sup>, 140 (1.9), 126 (7.4), 112 (37.1), 98 (100.0) [M - C<sub>4</sub>H<sub>9</sub>]<sup>+</sup>, 84 (26.4) [M - C<sub>5</sub>H<sub>11</sub>]<sup>+</sup>, 70 (19.0), 56 (13.7) [M - C<sub>5</sub>H<sub>11</sub> - C<sub>2</sub>H<sub>4</sub>]<sup>+</sup>, 41 (13.7).

## 2.6. Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analyses

Analyses of the essential oils and extracts were carried out by GC and GC-MS. The second method was also used to follow synthetic steps and in preliminary determination of the composition of reaction mixtures. The GC-MS analyses (three repetitions for each sample)

were performed on a Hewlett-Packard 6890N gas chromatograph equipped with fused silica capillary column DB-5MS (5% phenylmethylsiloxane, 30 m × 0.25 mm, film thickness 0.25 μm, Agilent Technologies, USA) and coupled with a 5975B mass selective detector from the same company. The injector and interface were operated at 250 and 290 °C, respectively. Oven temperature was raised from 70 to 290 °C at a heating rate of 5 °C/min and then isothermally held for 20 min. As a carrier gas He at 1.0 mL/min was used. The samples, 1 μL of the solutions in Et<sub>2</sub>O (*ca.* 1 mg in 1 mL of Et<sub>2</sub>O), were injected in a pulsed split mode (the flow was 1.5 mL/min for the first 0.5 min and then set to 1.0 mL/min throughout the remainder of the analysis; split ratio 40:1). Mass selective detector was operated at the ionization energy of 70 eV, in the 35-650 amu range and scanning speed of 0.34 s. The percentage composition was computed from the total ion chromatogram peak areas without the use of correction factors. Qualitative analyses of the essential oil, extract and reaction mixture constituents were based on the comparison of their linear retention indices (RI) relative to retention times of C<sub>7</sub>-C<sub>31</sub> *n*-alkanes on the DB-5MS column (Van den Dool and Kratz, 1963) with those reported in the literature, and by comparison of their mass spectra with those of authentic standards, as well as those from Wiley 6, NIST02, MassFinder 2.3, and a homemade MS library with the spectra corresponding to pure substances and components of known essential oils, and wherever possible, by co-injection with an authentic sample.

### 2.7. Acetylcholine-induced abdominal writhing test

Mice were treated according to Whittle (1964) and adapted by Matheus et al. (2005). Briefly, the total number of writhings following intraperitoneal (i.p.) administration of acetylcholine (ACh, 4 mg/kg) was recorded over a period of 20 min, starting 5 min after ACh injection.

Mice were pre-treated with conmaculatin (**1**), coniine or vehicle, 15 min before administration of ACh.

### 2.8. Hot-plate test

Mice were tested according to the method described by Sahley and Berntson (1979) and adapted by Matheus et al. (2005; Pinheiro et al., 2010). Animals were placed on a hot plate (Insight equipments, Brazil) set at  $55 \pm 1$  °C. Reaction time was recorded when the animals licked their fore- and hind-paws and jumped at several intervals of 30 min after i.p. administration of the ACh or morphine. Mice received i.p. injection of conmaculatin or coniine (10 or 20 mg/kg) 15 min before ACh injection. Baseline was considered as the mean of reaction time obtained at 60 and 30 min before administration of ACh or morphine and defined as normal reaction of the animal to the temperature. Increase in the baseline (%) was calculated by the formula:  $((\text{reaction time} - 100)/\text{baseline}) - 100$ .

### 2.9. Statistical analysis

All experimental groups were composed of 6-10 mice. The results are presented as mean  $\pm$  standard deviation. Statistical significance between groups was determined by the ANOVA analysis of variance followed by Tukey's test. *P* values less than 0.05 ( $P < 0.05$ ) were used as the significance level.

## 3. Results and discussion

### 3.1. Chemistry

Fresh plant material (fruits, above and underground parts), collected in two phenological stages, was extracted for essential oils and submitted to alkaline solvent extraction. The GC and GC-MS analyses of the obtained extracts and essential oils revealed the presence of well-known piperidine alkaloids, such as  $\gamma$ -coniceine, coniine and conhydrine, in all analyzed samples excluding the roots that were completely devoid of any alkaloids. A minor component (RI 1205) detected in the hydrodistilled fruit essential oils (going up to 2.5% of the oils), as well as in the fruit and both leaf and flower alkaline solvent extracts, caught our attention. The resemblance of mass spectral fragmentation patterns (the base peak at  $m/z$  84, as well as other fragment ions) of coniine (2-propylpiperidine, RI 1027,  $M^+$  ion at  $m/z$  127) and this unknown compound (RI 1205,  $M^+$  ion at  $m/z$  155) suggested that the mentioned compound possesses two extra  $-CH_2-$  groups, i.e. that it is possibly a dihomologue of coniine. These facts, along with the retention index increment of *ca.* 200 units, led us to tentatively identify this compound as 2-pentylpiperidine (**1**). A detailed literature survey showed that there were no previous reports on the occurrence of **1** in samples of natural origin. An additional argument strengthening our hypothesis was the fact that dihomologues of  $\gamma$ -coniceine and conhydrine, 2-pentyl-3,4,5,6-tetrahydropyridine and 5-hydroxy-2-pentylpiperidine, respectively, were found during the mentioned phytochemical reinvestigation of *C. maculatum* (Lang and Smith, 1998). This demonstrates the potential of this species to biosynthesize piperidine alkaloids with 5 acetate units. Having all of this in mind we decided to confirm our tentative structure assignment by comparing the chromatographic properties of this unknown constituent of *C. maculatum* to that of synthetic 2-pentylpiperidine, since the isolation of this unknown is virtually impossible due to the low yield (0.01% based on fresh weight) of complex extracts (hydrodistilled oils and solvent extracts) and its low relative abundance in the mixtures (< 3%).



A number of synthetic approaches to 2-alkyl substituted piperidines, especially coniine, have been pursued since they occupy a very important position both as bioactive targets (known to be inhibitors of the neuromuscular, ganglionic, central neuronal nicotinic acetylcholine receptors and HIV-protease (Uenishi et al., 2009)) and as a core structure in many other naturally occurring (Evans, 2001) or synthetic (Singh and Bisai, 2007) alkaloids. Moreover, as these alkaloids are usually available in only trace amounts from natural sources, there is a great need for diverse methods of their synthesis. Thus, so far, there have been several reported synthesis of 2-pentylpiperidine mostly as a part of the development of more general methods for the preparation of 2-alkyl substituted piperidine derivatives (Franke and Prodinge, 1931; Franke et al., 1936; Lukes and Janda, 1960; Pieper and Wegler, 1950; Royer et al., 2007) or as useful synthetic intermediate in the synthesis of some other more complex alkaloids (Daloze et al., 2000; Lukes and Cervinca, 1958). For our synthesis of 2-pentylpiperidine, we selected the strategy that relies on N atom insertion into a five member ring through Beckmann rearrangement of an appropriate oxime. Following this approach, the synthesis of racemic 2-pentylpiperidine was achieved in 8 reaction steps starting from cyclohexanol with the overall yield of 12% (as depicted in Scheme 1). Our route began with a two step transformation of the commercially available cyclohexanol (**2**) (oxidation to adipic acid and subsequent esterification of the obtained acid) to diethyl adipate (**3**) that was further submitted to standard Dieckmann condensation conditions (Vogel, 1998). The Dieckmann product (**4**) was alkylated with 1-iodopentane (**6**), using  $K_2CO_3$  in acetone, to yield ethyl 2-oxo-1-pentylcyclopentanecarboxylate (**7**) that was later transformed to 2-pentylcyclopentanone (**8**) by tandem acidic hydrolysis and decarboxylation (Ikan and Ravid, 1974). The conversion of ketone **8** to the appropriate oxime **9** was accomplished by simple grinding of the ketone with  $NH_2OH \cdot HCl$  and NaOH without solvent at room temperature (Vukićević et al., 2004). The oxime **9** submitted to Beckmann reaction conditions by heating

with 75% H<sub>2</sub>SO<sub>4</sub> (Hildebrand and Bogert, 1936), yielded a complex mixture, predominantly consisting of Beckmann rearrangement and fragmentation products, that was subjected to gradient MPLC (100% *n*-hexane to 100% Et<sub>2</sub>O, with the increment step of 5%). Pure 6-pentylpiperidin-2-one (**10**) obtained after chromatographic separation was finally reduced to **1** (2-pentylpiperidine) with LiAlH<sub>4</sub> in dry Et<sub>2</sub>O (Davies et al., 2004). Gas co-chromatography of the obtained standard with the fruit essential oil of *C. maculatum* unambiguously confirmed the original assumption.

The structural assignment of the target molecule as well as of the synthetic intermediates, and some by-products, was achieved by spectral means (1D and 2D NMR, IR, MS). GC-MS analysis of the synthesized 2-pentylpiperidine gave a peak in the total ion chromatogram at RI 1205 and a parent ion [M]<sup>+</sup> at *m/z* 155 (C<sub>10</sub>H<sub>21</sub>N) in its MS. The <sup>13</sup>C NMR spectrum contained the expected number of signals for compound **1** (ten signals). Two of them at δ 56.8 and 47.0, assignable to C-2 and C-6 atoms, respectively, probably directly attached to the nitrogen atom, a wide IR band at 3200-3500 cm<sup>-1</sup>, together with a decrease in the integral value of 1 for a <sup>1</sup>H NMR signal at 2.44-2.74 ppm, upon the addition of D<sub>2</sub>O, suggested the presence of a secondary, strongly hydrogen bonded, amine group. The base peak ([M - C<sub>3</sub>H<sub>11</sub>]<sup>+</sup>) in its MS at *m/z* 84, as well as the ion [M - H]<sup>+</sup> at *m/z* 154 (the characteristic α-cleavage of amines), were indicative of a pentyl side chain in the α position. The formed iminium ion loses ethene (by *retro*-Diels-Alder) and this is consistent with the specific fragmentation pattern of the piperidine ring (Barker and Ando, 1998). The <sup>1</sup>H and <sup>13</sup>C NMR data matched those already reported (Daloze et al., 2000; Royer et al., 2007). No previous attempt of an assignation of Hs and Cs NMR signals of **1** was made. We partially assigned the NMR data (Table 1) by the use of <sup>1</sup>H - <sup>1</sup>H COSY and HETCOR spectra and comparison with literature data for an acyclic related secondary amine (Djerassi and Eggert, 1973). The HETCOR spectrum showed connections between C-2 and a multiplet proton signal at 2.24-

2.42 ppm, whereas C-6 was coupled to a proton at 2.90-3.04 ppm and with one of the two protons from the signal at 2.44-2.74 ppm. Also, the HETCOR spectrum pointed out to the presence of additional three diastereotopic pairs of Hs (besides the -C6-H<sub>2</sub>-) that most probably belong to the ring methylenes since the free rotation should make the other -CH<sub>2</sub>- in the side chain isochronous. In this way the signals at 32.7, 26.4 and 24.7 ppm were assigned to the other Cs of the piperidine ring while the remaining four side chain -CH<sub>2</sub>- groups gave signals at 37.2, 31.9, 25.4 and 22.4 ppm. The instantly apparent triplet at  $\delta$  0.79 in <sup>1</sup>H-NMR of the CH<sub>3</sub> group correlated in the HETCOR to the signal at  $\delta$  13.8 in the <sup>13</sup>C-NMR spectrum. This connectivity pattern was conformed by the cross peaks observable in the COSY spectrum. Unfortunately, the information obtained from these 2D NMR spectra allowed only the assignation of the piperidine ring Cs whereas the assignation of those of the side chain required further matching of the unassigned chemical shifts to those of an analogue's Cs in *N*-butylheptan-2-amine (Djerassi and Eggart, 1973). However, still unresolved remain the side chain Hs due to strong overlap in the narrow range of 1.15-1.35 ppm.

Prolonged storage of conmaculatin in aerated CDCl<sub>3</sub> at 25 °C produced no visible alterations in the appearance of its <sup>1</sup>H-NMR spectrum or the quantity of this compound (as inferred from the addition of an internal standard). The same was noted for coniine, but the signals for -coniceine became indiscernible against the newly formed background after just one week of storage.

### 3.2. Biology

Encouraged by the findings of previous studies on biological activity of common hemlock alkaloids, especially coniine, which among other things, acts as an antinociceptive agent (Arihan et al., 2009; Bowman and Sanghvi, 1963), we decided to test the obtained synthetic

sample of compound **1** for peripheral and central antinociceptive activity in two models (effects on acetylcholine-induced writhings and the hot plate test, in mice). In both models the antinociceptive effect (Fig. 1) of the compound was observed in a very narrow dose range (10-20 mg/kg) with no detectable activity below 10 mg/kg and being lethal in doses over 20 mg/kg. When compared to coniine, which induced fasciculations, dyskinesia, convulsions and death, at 25 mg/kg, conmaculatin (**1**) can be considered of more or less the same toxicity. Both coniine and conmaculatin showed a dose dependent antinociceptive effect in 10 to 20 mg/kg (sublethal) doses (Arihan et al., 2009).

The results of the acetylcholine-induced writhings model showed that the administration of conmaculatin at 10 mg/kg produced a 0.5 point decrease in the number of writhes (which is the quantitative criterion for the existence of nociception) while the treatment with 20 mg/kg resulted in about 60% inhibition of the number of writhes compared to the control group ( $P < 0.05$ , Fig. 1). For comparison sake, one should have in mind that the decrease in the number of writhes promoted by the standard drug morphine at 0.3 mg/kg was 3.5 points. At the same dose, the antinociceptive activity exhibited by conmaculatin was quantitatively similar (the decrease in the number of writhes in the chemical pain test) to the one published for coniine (Arihan et al., 2009). It is well known that acetylcholine is a pain inducer. Accordingly, the i.p. injection of ACh induces an effect that is quantified as evoking a shorter response of animals to thermal stimulation. The administrations of conmaculatin (20 mg/kg) 15 min prior to ACh resulted in a reversal of the effect induced by ACh. The activity of conmaculatin is also qualitatively different from the one for morphine (data not shown). It is well known that morphine exerts its antinociceptive effect through central opiate receptors (Guyton and Hall, 2001). Recently, in both *in vitro* and *in vivo* pharmacodynamic studies on human fetal-muscle cells and mice, Lee et al. (2008; Green et al., 2010) showed that coniine binds to the nicotinic-type acetylcholine receptor. Due to its resemblance to coniine, it is possible that

conmaculatin also manifests its antinociceptive effect *via* the nicotinic receptors. Moreover, neuronal nicotinic acetylcholine receptor agonist nicotine has long been known to have antinociceptive effects in both experimental animals and humans. In animal studies, however, high doses of nicotine are required to produce antinociception, and this effect is relatively modest and of short duration (Decker and Meyer, 1999). Meanwhile, further investigation is required on the specific mode of action of conmaculatin in order to rationalize whether the activity of this alkaloid is through nicotinic receptors or if it also involves muscarinic receptors.

#### 4. Conclusions

The minor volatile component (RI 1205) detected in the hydrodistilled fruit essential oils, as well as in the fruit and both leaf and flower alkaline solvent extracts, of the well known weed plant species *C. maculatum* was identified as 2-pentylpiperidine by gas co-chromatography of the synthetic 2-pentylpiperidine with an oil sample. This is the first report on the occurrence of 2-pentylpiperidine in samples of natural origin and therefore it can be regarded as a new naturally occurring secondary plant metabolite (alkaloid) representing a dihomologue of coniine (one extra “acetate” unit compared to coniine, i.e. ten-carbon atom precursor produced by the combination of five acetate units). It is our proposal to name this compound conmaculatin.

The obtained synthetic sample of conmaculatin was screened for antinociceptive activity in peripheral and central analgesic models. Conmaculatin showed an antinociceptive effect in a dose dependent manner in the dose range 10-20 mg/kg. This is comparable to the one previously published for the other piperidine hemlock alkaloid, coniine.

Finally, the results of this study could also be of interest in a historical sense, since conmaculatin, together with coniine and other alkaloids from *C. maculatum*, could be responsible for the death of the famous Greek philosopher Socrates.

### **Conflicts of Interest**

The authors declare that there are no conflicts of interest.

### **Acknowledgements**

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### **Appendix A. Supplementary data**

Supplementary data associated with this article can be found in the online version.

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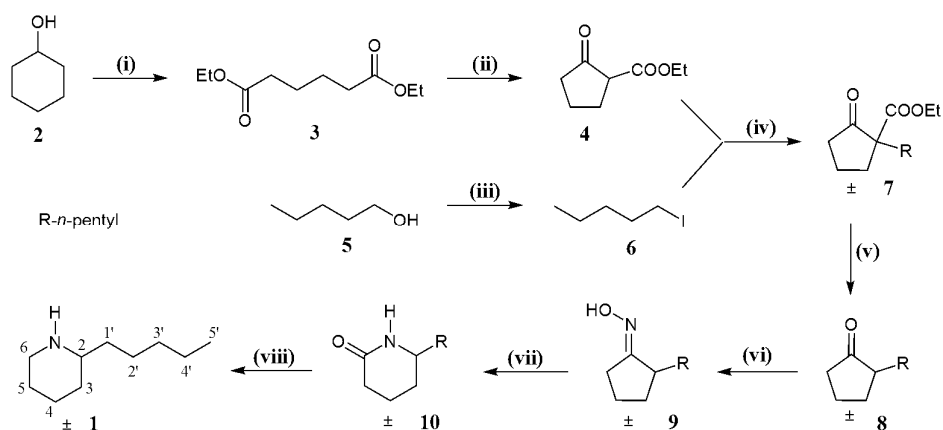
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**Scheme 1.** Reagents and conditions: (i) a) 9 eq HNO<sub>3</sub> (conc.), reflux, 5-6 h, b) 6 eq ethanol (anhydrous), toluene, H<sub>2</sub>SO<sub>4</sub> (cat.), 75-78 °C, overall yield 85%; (ii) 1.5 eq Na, ethanol (cat.), benzene, reflux, 8 h, yield 54%; (iii) 0.5 eq red P, 0.5 eq I<sub>2</sub>, reflux, 2 h, yield 67%; (iv), 4 eq K<sub>2</sub>CO<sub>3</sub> (anhydrous), acetone, reflux, 2 h, yield 93%; (v) 13.5 eq glacial acetic acid, 8.5 eq HCl (20% aq.), reflux, 24 h, 69%; (vi) 2.4 eq NaOH, 2.4 eq NH<sub>2</sub>OH·HCl, room temperature, 72 h, solvent-free condition, yield 98%; (vii) 3 eq H<sub>2</sub>SO<sub>4</sub> (75% aq.) dropwise, 108 °C, yield 57%; (viii) a) 5 eq LiAlH<sub>4</sub>, Et<sub>2</sub>O, reflux, 16 h, b) KOH (aq.), overall yield 75%.

**Table 1**<sup>1</sup>H and <sup>13</sup>C NMR and <sup>1</sup>H - <sup>1</sup>H COSY spectroscopic data for compound **1**<sup>a</sup>

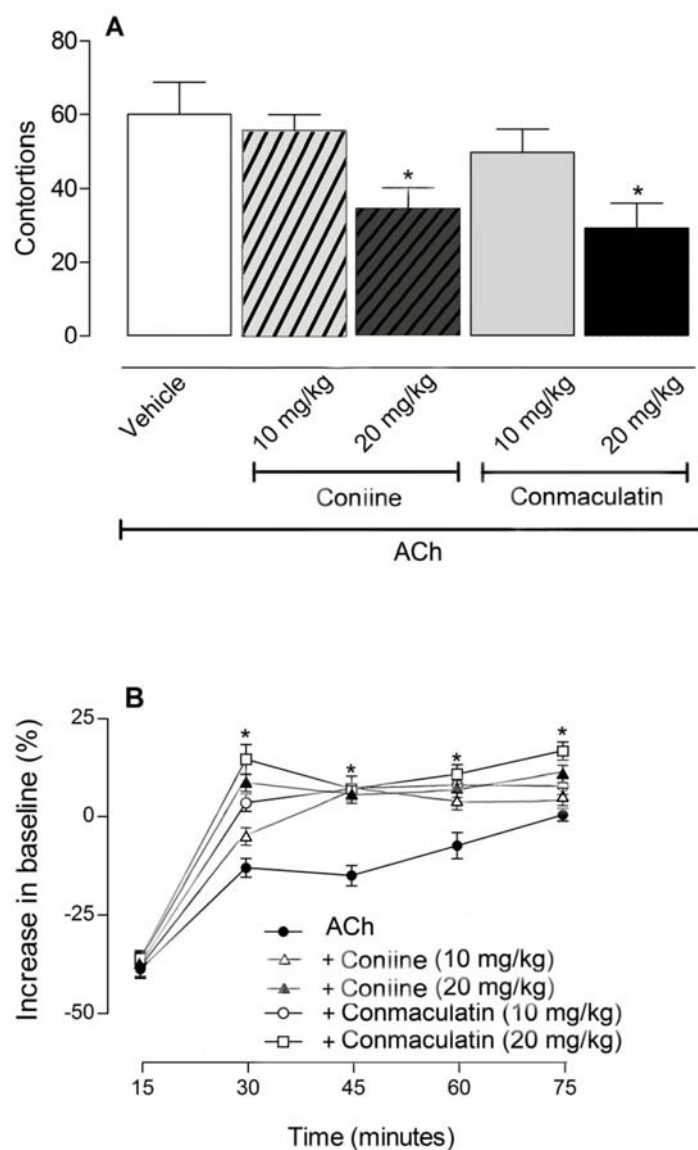
Position	$\delta_C$ (ppm)	$\delta_H$ (ppm)		<sup>1</sup> H- <sup>1</sup> H COSY
2	56.8	2.24-2.42 <i>m</i>		2Ha, 2Hb
3	32.7	a	$\approx 0.95^b$	1H, 2Hb
		b	$\approx 1.50^b$	1H, 2Ha
4	24.7	a	$\approx 1.20^b$	/
		b	$\approx 1.65^b$	/
5	26.4	a	$\approx 1.20^b$	4Hb, 5H <sub>eq</sub>
		b	$\approx 1.50^b$	4Ha, 5H <sub>eq</sub>
6	47.0	<i>ax</i>	2.44-2.74 <i>ddd</i> (3.0, 11.6, 14.8) <sup>c</sup>	5H <sub>eq</sub>
		<i>eq</i>	2.90-3.04 <i>m</i>	4Ha, 4Hb, 5H <sub>ax</sub>
1	37.2 <sup>d</sup>	$\approx 1.15$ -1.35 <sup>b</sup>		/
2	25.4 <sup>d</sup>	$\approx 1.15$ -1.35 <sup>b</sup>		/
3	31.9 <sup>d</sup>	$\approx 1.15$ -1.35 <sup>b</sup>		/
4	22.4 <sup>d</sup>	$\approx 1.15$ -1.35 <sup>b</sup>		5 H
5	13.8	0.79 <i>t</i> (6.5)		4 H
N-H	/	2.44-2.74 <i>m</i> <sup>c</sup>		/

<sup>a</sup> <sup>1</sup>H and <sup>13</sup>C NMR spectra were measured in CDCl<sub>3</sub> at 200 and 50 MHz, respectively; the coupling constants (*J*) are in parentheses and reported in Hz.

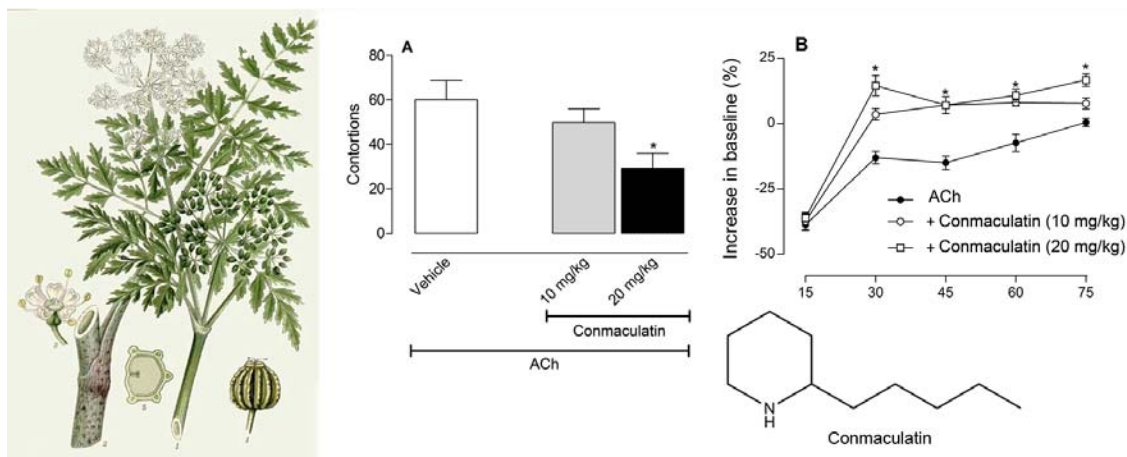
<sup>b</sup> The chemical shift approximate value determined from the HETCOR spectrum (part of the same overlapping multiplet signal at 0.90-1.72 ppm consisting of 14 protons)

<sup>c</sup> Overlapping signals

<sup>d</sup> Assigment was accomplished by comparison with literature data (Djerassi and Eggert, 1973)



**Fig. 1. A** - Effects of conmaculatin and coniine on acetylcholine-induced writhings in mice; **B** - Hot-plate test results. Control groups were treated with the vehicle and acetylcholine (ACh, 4 mg/kg, i.p.). All experimental groups were composed of 6-10 mice, and each experiment repeated five times. Statistical significance between groups was determined by the ANOVA analysis of variance followed by Tukey's test. \* $P < 0.05$  when comparing a treated group with a vehicle-treated group.



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- 2-Pentylpiperidine, named conmaculatin, was identified from the essential oil of *Conium maculatum* L.
- The new alkaloid was synthesized in 8 steps from cyclohexanol and in 12% total yield
- The biosynthesis of the alkaloid should follow the acetate pathway, known for coniine
- Conmaculatin was found to be lethal to mice in doses higher than 20 mg/kg
- 2-Pentylpiperidine showed a strong antinociceptive effect in a dose dependent manner

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