Phytochemical Study and Antiinflammatory Properties of *Lobelia laxiflora* L.

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Three new piperidine alkaloids were isolated from stems, leaves and flowers of *Lobelia laxiflora* L. (Campanulaceae). The structures of *racem. cis*-8,10-diethyl-3,4-dehydrolobelidiol (**1**), *racem. trans-8-ethyl-10-phenyl-3,4-dehydrolobelidiol* (**2**) and *racem. cis-8-ethyl-10-phenyl-3,4-dehydrolobelidiol* (**3**) were established by spectral analyses. The residues obtained from the ethanol extracts from stems (S), leaves (L), and flowers (F) were applied in carrageenan (Car)- and cobra venom (CV)-induced acute inflammation in mice. A suppression of paw edema formation at a dose of 100 mg kg^{-1} was established. In this study the antiinflammatory potential of *Lobelia l.* was regarded in connection with the complement system. The sequential activation and assembly into functional units of complement components can proceed via two different pathways, named classical (CP) and alternative (AP). The ability of the residues, nonalkaloid fractions, alkaloid fractions and the three alkaloids at a concentration from 0.125 to 1.0 mg ml^{-1} to inhibit complement activation and thus to prevent inflammatory process was estimated *in vitro* in human serum via both pathways. All of them inhibited complement activity with a predominant action on CP.

Introduction

Genus *Lobelia* (Campanulaceae) is represented by more than 15 species rich in piperidine alkaloids (Boit, 1961). *Lobelia inflata* L. is the most investigated species belonging to this genus, which is widely distributed in North America and cultivated in the Netherlands, Poland and Russia (Wagner, 1988). More than 20 alkaloids have been isolated from this plant, the major one being lobeline (Marion, 1950, Marion, 1960). In official medicine lobeline is administered as hydrochloride in the cases of asphyxia and poisoning with narcotics, barbiturates, etc. (Assenov and Nikolov, 1988). *Lobelia laxiflora* L. is a perennial blooming bush, 1 m in height, spread in Central America. In the folk medicine of Costa Rica plant decoctions are used for treatment of gastro-intestinal disorders. To our knowledge no chemical investigations of this species have been reported. The present study describes the phytochemical investigation of stems, leaves and flowers from *L. laxiflora* and the isolation and structural elucidation of three new piperidine alkaloids. The antiinflammatory property of the plant was evaluated in two models of paw edema in mice. The complement system is one of the major factors for generation of acute inflammatory process and its modulation by complement inhibitors appears to be crucial in such disorders. Two activation pathways have been characterized in the complement system. The classical pathway is triggered by IgG and IgM containing immune complexes, while the alternative pathway is activated by plant, fungal and bacterial polysaccharides. The anticomplement potency of the residues, fractions and pure alkaloids was estimated in human serum via CP and AP. Aggregated IgG and fungal polysaccharide zymosan are widely used as complement activators with defined mode of action for *in vitro* and *in vivo* studies, the former in CP assays and the second one in AP assays (Autery *et al.*, 1988; Whaley *et al.*, 1975). Their inhibitory effect was compared with this expressed by *Lobelia* solutions.

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Materials and Methods

General experimental procedures

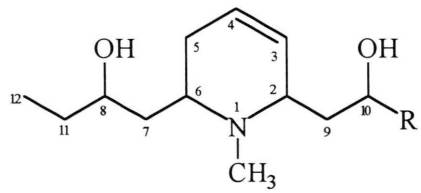
IR spectra were taken on a Specord 75 IR spectrometer. The EI- and CI-mass spectra were recorded on a Varian MAT 311A spectrometer. ^{1}H- and ^{13}C-NMR including DEPT 135 and 2D-NMR spectra were recorded on a Bruker DRX 250 MHz spectrometer in $CDCl_3$ and chemical shifts are given in δ (ppm) with TMS as internal standard. The NOE difference spectra were measured by the use of a standard Bruker software program. Optical rotation was measured on a Perkin-Elmer 241 polarimeter. Silica gel 60 (30–70 mesh, Merck) was used for column chromatography (CC), silica gel 60 H (Merck) for short column chromatography (SCC), silica gel 60 PF_{254} for preparative TLC and aluminum sheets silica gel 60 F_{254} for TLC. Compounds were visualized by spraying with Dragendorff's reagent.

Plant material

Lobelia laxiflora was collected in Costa Rica in the mountain "Zerro de la Muerte", in January of 1995 during the time of flowering and separated into stems, leaves and flowers. A voucher specimen (No 75130) was deposited at the herbarium of the National Museum of Costa Rica.

Extraction and isolation

The air-dried and ground plant material of *Lobelia laxiflora*, separated into stems (S=680 g), leaves (L=340 g) and flowers (F=100 g) was extracted with a mixture 95% EtOH-H_2O (9:1, v/v) at room temperature – 6x3 l for S and L and 6x1 l for F. The corresponding extracts were concentrated under reduced pressure and the residues (60 g from S, 36 g from L and 15 g from F) were acidified with 3% HCl (50 ml) and left overnight at room temperature. The insoluble materials were filtered and the non-alkaloid fractions (4.06 g from S, 2.04 g from L and 1.18 g from F) were obtained. Then acidic solutions were made alkaline with 25% NH_4OH (pH 9–10) and extracted with $CHCl_3$ (6x100 ml). The $CHCl_3$ extracts were combined, dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to give the crude alkaloid fractions (0.31 g from S, 0.70 g from L and 0.17 g from F). These alkaloid fractions were further separated by the use of silica gel column chromatography, eluted with $CHCl_3$-MeOH mixtures of increasing polarity and 6 combined fractions were obtained from S and 3 from L and F. Each of these fractions were subjected to silica gel short column chromatography, eluted with hexane-Me_2CO mixture (1:1, v/v). Finally, the isolated crude alkaloids were purified by preparative TLC (petroleum ether-$CHCl_3$-ME_2CO-MeOH, 2.5:8:1:2) to yield three pure alkaloids: from S – **1** (9.0 mg), **2** (2.1 mg) and **3** (1.9 mg); from L – **1** (25.0 mg), **2** (2.2 mg) and **3** (2.5 mg); from F – **1** (10.0 mg), **2** (1.0 mg) and **3** (1.7 mg).



1 R = $-CH_2CH_3$

2 and **3** R =

racem. cis-8,10-diethyl-3,4-dehydrolobelidiol (**1**): Resinous substance; $[\alpha]_D$ 0° (c 0.18, EtOH); IR (film) ν_{max} (cm^{-1}): 3400 (br., O-H), 1640 (C=C). CI-MS *m/z* (rel. int.%): 242 $[M+1]^+$ (100); EI-MS *m/z* (rel. int.%): 241 $[M+1]^+$(2), 226 (2), 212 (10), 168 (100), 108 (6), 94 (50), 59 (8), 42 (8), 29 (4). ^{1}H- and ^{13}C-NMR (Table I).

racem. trans-8-ethyl-10-phenyl-3,4-dehydrolobelidiol (**2**): Resinous substance; $[\alpha]_D$ 0° (c 0.07, EtOH); IR (film) ν_{max} (cm^{-1}): 3400 (br., O-H), 1660 (C=C), 1600, 1500, 1450 (benzene ring); CI-MS *m/z* (rel. int.%): 290 $[M+1]^+$ (100); EI-MS *m/z* (rel. int.%): 289 $[M]^+$(10), 260 (10), 216 (100), 182 (5), 168 (50), 94 (65). ^{1}H- and ^{13}C-NMR (Table II).

racem. cis.-8-ethyl-10-phenyl-3,4-dehydrolobelidiol (**3**): Resinous substance; $[\alpha]_D$ 0° (c 0.11, EtOH); IR (film) ν_{max} (cm^{-1}): 3400 (br., O-H), 1650 (C=C), 1600, 1500, 1450 (benzene ring); CI-MS *m/z* (rel. int.%): 290 $[M+1]^+$ (100); EI-MS *m/z* (rel. int.%): 289 $[M]^+$(10), 260 (10), 216 (100), 182 (5), 168 (50), 94 (65). ^{1}H- and ^{13}C-NMR (Table II).

Carrageenan- and CV-induced paw edema in mice

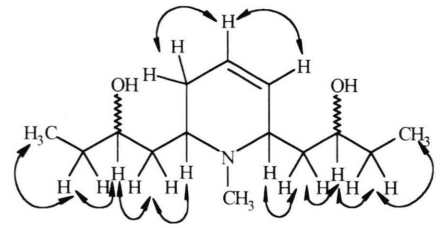
A volume of 0.05 ml of 1% carrageenan (Fluka) or 0.05 ml of CV-solution (1 μg/paw) was injected into the right hind paw of mice. The left hind paw was injected with 0.05 ml of saline and served as control. Mice were killed after 3.5 h (Car-edema) or after 2.5 h (CV-edema), both hind paws were cut and weighed. As reference substances acetylsalicylic acid (ASA) in carrageenan edema and indomethacin (IM, Sigma Chemicals Co, St. Louis) in CV-edema were used.

Complement assay

The *in vitro* effect of different residues and fractions on CP and AP activity was determined in normal human serum (NHS) by microtiter assay as described by Klerx *et al.* (1983). For the CP, veronal buffer saline (25 mM, pH 7.4), containing 0.15 mM Ca^{2+}, 0.5 mM Mg^{2+} and 0.05% gelatine (GVB^{2+}) was used as diluent and sensitized sheep erythrocytes at a concentration of 2 x 10^8 cells ml^{-1} served as target. For the AP, veronal containing 0.25 mM Mg^{2+}, 8 mM ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid, and 0.05% gelatine (GVB-EGTA) was used as diluent and nonsensitized rabbit erythrocytes at a concentration of 1 x 10^8 cells ml^{-1} were used as target. Human serum was diluted to 1:40 in the CP assay and in AP assay to 1:4.5, in order to give 50% haemolysis. After preincubation of 0.1 ml of NHS with 0.1 ml of different concentrations of the solutions, 0.05 ml of the appropriate target erythrocytes were added and after 60 min (CP assay) or 45 min (AP assay) at 37^0C the haemolysis in the supernatants was determined spectrophotometrically at 405 nm. Heat aggregated human IgG were prepared by incubating 10 mg ml^{-1} IgG at 63^0C for 20 min. Particulate aggregates were removed by centrifugation and they were added as 1% suspension in GVB^{2+} (0.05 ml) to 0.1 ml NHS. Activated zymosan (Zy, from *Saccharomyces cerevisiae*, Sigma Chemical Co, St. Louis, USA) after boiling 30 min in saline was added as 1% suspension in GVB-EGTA to NHS. Percentages of inhibition were calculated and compared to those of the control samples where buffer instead of substances was added (0% inhibition).

Results and Discussion

The main alkaloid isolated as a resinous substance from the three plant parts investigated is *racem. cis*-8,10-diethyl-3,4-dehydrolobelidiol (**1**). The ion peak at *m/z* 242 $[M+H]^+$ in the CI-mass spectrum corresponds to the molecular formula $C_{14}H_{27}NO_2$. The base peak at *m/z* 168 $[M\text{-}C_2H_5CH(OH)CH_2]^+$ and the peak at *m/z* 94 $[C_5H_5NCH_3]^+$ in the EI-mass spectrum which were obtained after cleavage of the one or the both side chains, respectively, confirmed this structure. The fragments at *m/z* 226 $[M\text{-}CH_3]^+$, 212 $[M\text{-}C_2H_5]^+$, 108 $[M\text{-}C_2H_5CH(OH)CH_2\text{-}C_2H_5CH(OH)]^+$, 59 $[C_2H_5CH(OH)]^+$ and 42 $[59\text{-}OH]^+$ which are characteristic for the mass spectral fragmentation of *Lobelia* alkaloids were also observed (Spitteler-Friedman and Friedman, 1965). The ^{1}H-NMR spectrum of **1** shows a dd signal at δ 5.45 and a multiplet at δ 5.80–5.88, which are for the two protons from the C-3, C-4 double bond in the piperidine ring. The absorption at 1640 cm^{-1} in the IR spectrum also confirms this fact. The ^{1}H-NMR spectrum of **1** revealed the presence of two OH groups at δ 5.25 (broad two-proton singlet, disappeared after addition of D_2O). They are also observed as a broad signal at 3400 cm^{-1} in the IR spectrum. The proposed structure of **1** was confirmed by the ^{13}C-NMR spectrum and the DEPT 135 experiment. The carbons carrying the OH groups (C-8 and C-10) as well as the carbons connected with the nitrogen atom (C-2 and C-6) appeared at δ 71.0, 71.7, 60.8 and 60.0, respectively. The carbons from the double bond were observed at δ 125.5 and 125.6. The data of ^{1}H,^{1}H-COSY and HMQC experiments enabled the location of all the hydrogen and carbon atoms (data are summarized in Fig. 1 and Table I). The assignment of the relative configuration at C-2 and C-6 was made by the use of NOE difference experiments. Irradiation of H-2 (δ 4.12–4.25) created 6.2% NOE of H-6 (δ 3.61–3.69) and irradiation of H-6 (δ 3.61–3.69) led to 8.1% NOE of H-2 (δ 4.12–4.25). These results determined directly the *cis* axial position between H-2 and H-6. The Dreiding stereomodels also show that only at this situation between H-2 and H-6 the NOE effects could be observed. Irradiation of NCH_3 (δ 2.46) group does not give any NOE of H-2 and H-6, which additionally confirms the *cis* axial relative



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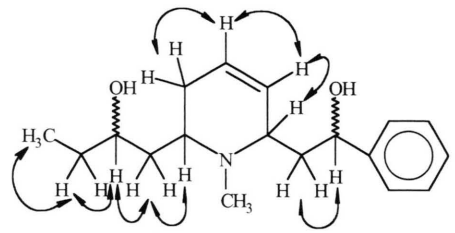

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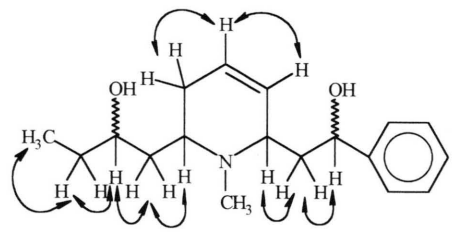

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Fig. 1. $^1H,^1H$-COSY correlations.

Table I. ^1H- and ^{13}C-NMR chemical shifts of **1** according to HMQC.

C-atom	δC	δH (J, Hz)
1	24.1	2.46, s
2	60.8	4.12–4.25, m
3	125.6	5.45, dd (1.9, 10.2)
4	125.5	5.80–5.88, m
5	26.1	2.02–2.20, m
6	60.0	3.61–3.69, m
7	37.1	1.72–1.89, m 1.40–1.60, m
8	71.0	3.72–3.85, m
9	37.3	1.72–1.89, m 1.40–1.60, m
10	71.7	3.72–3.85, m
11	30.9	1.40–1.60, m
12	9.7	0.93, t (7.4)
13	31.0	1.40–1.60, m
14	9.7	0.95, t (7.4)

configuration. Compound **1** is racemate, since $[\alpha]_D$ is 0°. According to the above data the structure of the new alkaloid **1** is elucidated as *racem. cis*-8,10-diethyl-3,4-dehydrolobelidiol.

The other two alkaloids **2** and **3** have completely identical mass spectra. The ion peak at *m/z* 290 $[M+H]^+$ in the both CI-mass spectra corresponds to the molecular composition $C_{18}H_{27}NO_2$. The base peak at *m/z* 216 $[M\text{-}C_2H_5\text{-}CH(OH)CH_2]^+$ and this at *m/z* 182 $[M\text{-}C_6H_5CH(OH)CH_2]^+$ in the EI-mass spectra of **2** and **3** indicated the two side chains of the compounds. The rest of fragments (see Experimental) in the both spectra displayed the typical fragmentation for *Lobelia* alkaloids (Spiteller-Friedman and Spiteller, 1965). The ^1H-NMR spectra of **2** and **3** are very similar and like the spectrum of **1** showed signals for one double bond in piperidine ring – a dd signal at δ 5.59 and a multiplet at δ 5.77–5.81 for **2** and a dd signal at δ 5.46 and a multiplet at δ 5.81–5.89 for **3**. The double bond in **2** and **3** is confirmed from the IR absorptions at 1660 and 1650 cm^{-1}, respectively. The broad singlet for two protons (disappeared after addition of D_2O) at δ 3.40 for **2** and at δ 3.36 for **3** indicated the presence of two OH groups. The absorption at 3400 cm^{-1} in both IR spectra supported this fact. The ^{13}C-NMR spectrum as well as the DEPT 135 of **2** is related to same experiments of **3**. The atoms C-2 and C-6 appeared at δ 62.9 and δ 50.1 for **2** and at δ 60.1 and δ 61.1 for **3**. The signals at δ 73.7 and δ 72.6 for **2** and these at δ 71.4 and δ 72.8 for **3** corresponded to C-8 and C-10 atoms, carrying the OH groups. The carbons from the double bond (C-3 and C-4) were observed at δ 127.5 and 124.7 for **2** and at δ 125.6 and 125.4 for **3**, respectively. The data of $^1H,^1H$-COSY and HMQC experiments enabled the location of all the hydrogen and carbon atoms (data are summarized in Fig. 1 and Table II). The data described for **2** and **3** displayed structure 8-ethyl-10-phenyl-3,4-dehydrolobelidiol. The differences in the R_f values, as well as in the data of the NOE dif experiments indicated that the alkaloids **2** and **3** are diastereomers. About **2** any NOE between H-2 and H-6 was not observed. The irradiation of NCH_3 (δ 2.47) gives 1.2% NOE only of H-6 (δ 3.42–3.48). From these data, as well as from the Dreiding stereomodels could be proved the trans-axial-equatorial relationship of H-2 and H-6. Since, $[\alpha]_D$ is 0^0 the structure of the new alkaloid

Table II. ^{1}H and ^{13}C-NMR chemical shifts of **2** and **3** according to HMQC.

C-atom	**2** δC	**2** δH (J, Hz)	**3** δC	**3** δH (J, Hz)
1	34.6	2.47, s	27.3	2.51, s
2	62.9	3.33–3.37, m	60.1	3.71–3.81, m
3	127.5	5.59, dd (1.8, 10.2)	125.6	5.46, dd (1.7, 10.2)
4	124.7	5.77–5.81, m	125.4	5.81–5.89, m
5	24.5	1.98–2.02, m	26.1	2.02–2.42, m
6	50.1	3.42–3.48, m	61.1	4.21–4.29, m
7	38.2	1.48–1.75, m	37.2	1.47–1.83, m
8	73.7	3.74–3.88, m	71.4	3.87–3.97, m
9	41.6	1.98–2.02, m	40.1	2.02–2.42, m
		1.48–1.74, m		1.47–1.83, m
10	72.6	4.86, dd (3.2, 10.8)	72.8	5.07, dd (3.1, 10.6)
11	30.8	1.48–1.75, m	30.8	1.47–1.83, m
12	9.9	0.97, t (7.4)	9.7	0.97, t (7.4)
1'	145.0	–	144.3	–
2'	128.4	7.34–7.44, m	128.3	7.36–7.40, m
3'	125.8	7.34–7.44, m	127.3	7.36–7.40, m
4'	126.7	7.34–7.44, m	128.3	7.36–7.40, m
5'	125.8	7.34–7.44, m	127.3	7.36–7.40, m
6'	128.4	7.34–7.44, m	128.3	7.36–7.40, m

2 is determined as *racem. trans-8-ethyl-10-phenyl-3,4-dehydrolobelidiol.* The irradiation of H-2 (δ 3.71–3.81) in **3** created 4.3% NOE of H-6 (δ 4.21–4.29) and the irradiation of H-6 (δ 4.21–4.29) led to 5.2% NOE of H-2 (δ 3.71–3.81). The irradiation of NCH_3 (δ 2.51) does not give any NOE of H-2 (δ 3.71–3.81) and H-6 (4.21–4.29) and as in **1** the position between H-2 and H-6 is cis-diaxial. Alkaloid **3** is a racemate, since $[\alpha]_D$ is 0^0. The combination of these data elucidated the structure of the new alkaloid **3** as *racem.cis-8-ethyl-10-phenyl-3,4-dehydrolobelidiol.*

The antiinflammatory effect of the three residues was tested in two models of acute inflammation. They were applied i.p. at a dose of 100 mg kg^{-1} in carrageenan- and cobra venom-induced paw edema (Fig. 2). The flower residue suppressed edema formation caused by carrageenan most strongly (50% inhibition) as compared to the residues from stems and leaves (30% inhibition). ASA at a dose of 100 mg kg^{-1} expressed 38% inhibition of paw swelling. In CV-induced inflammation the reducing effect of flower residue (75% inhibition) was comparable to the effect of 10 mg kg^{-1} indomethacin (85% inhibition). The residues from leaves and stems were less effective (40% and 35% inhibition, respectively).

The results from determining complement activity via CP showed that the three residues strongly inhibited hemolysis at a concentration range be-

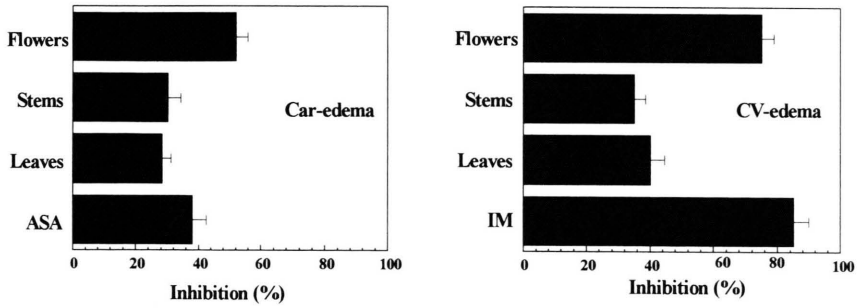

Fig. 2. Inhibition of carrageenan- and cobra venom-induced paw edema in mice. Residues from different plant parts were applied i.p. 30 min before the phlogistic agents at a dose of 100 mg kg^{-1}. Data are means ± SD from groups of 7 mice; **p<0.01, Student's *t*-test.

tween 0.125 and 1.0 mg ml^{-1} (Fig. 3). The highest effect expressed the flower residue. Nonalkaloid fractions from leaves and flowers showed similar inhibitory activity (from 40% up to 70% inhibition), while stem fraction was with higher activity (from 40% up to 90% inhibition). Alkaloid fractions were less active in comparison to residues and nonalkaloid ones and no significant differ-

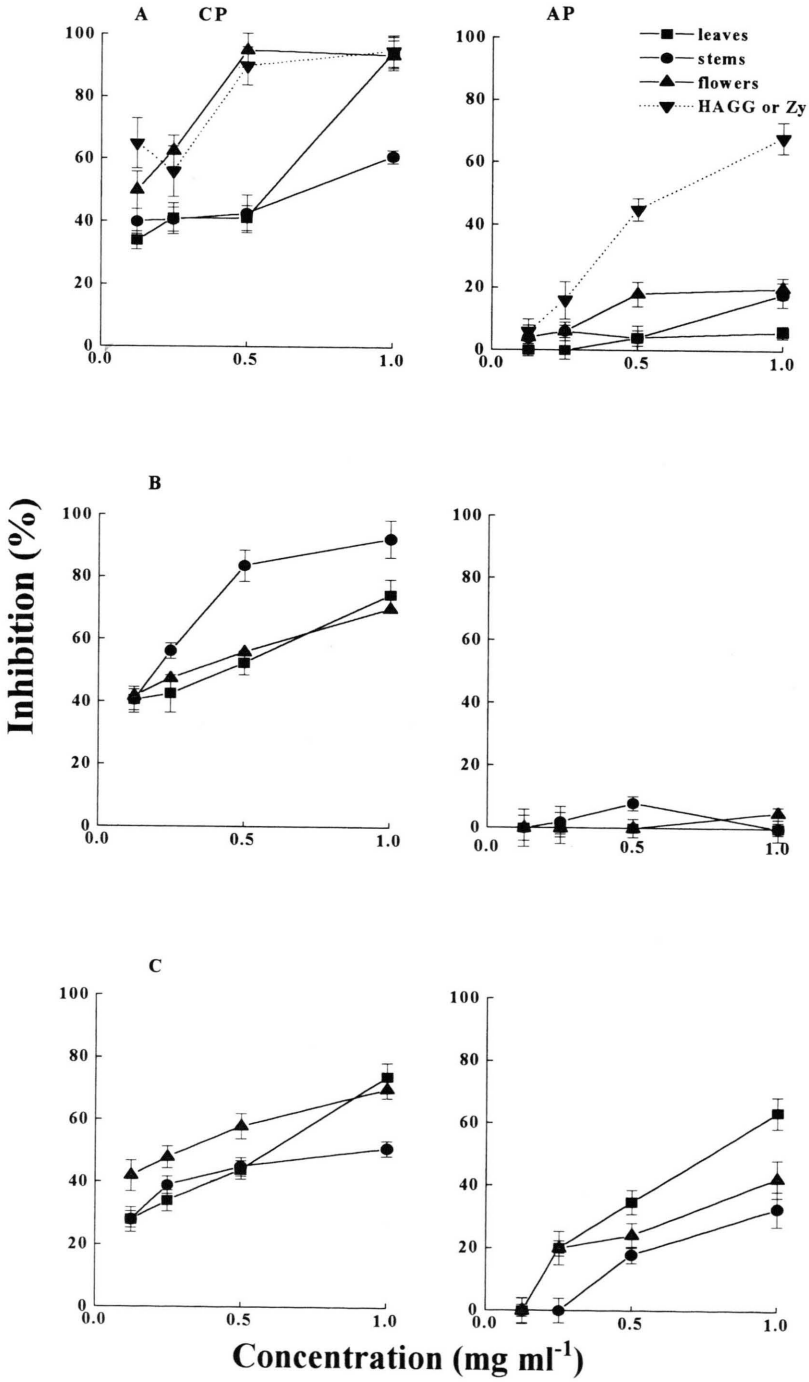

Fig. 3. Inhibition of classical pathway (CP) and alternative pathway (AP) activity in NHS by the residues (**A**), nonalkaloid fractions (**B**) and alkaloid fractions (**C**). Data represent means ± SD from three determinations. **p<0.01, Student's *t*-test.

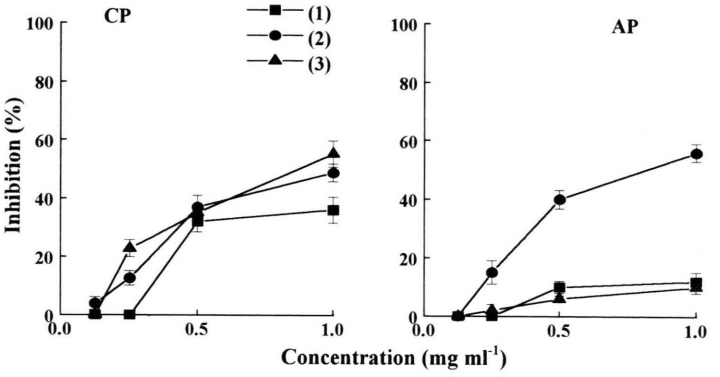

Fig. 4. Effect of the alkaloids on CP and AP activity in NHS. Data represent means ± SD from three determinations.

ences among them were observed. The effect of the flower residue and nonalkaloid fraction from stems was comparable with the effect expressed by the aggregated IgG, which powerfully can activate complement system via CP. Relating to AP activity the residues from stems and flower caused weak suppression only at the highest concentration of 1 mg ml^{-1}. Nonalkaloid fractions were completely ineffective and alkaloid fractions decreased moderately AP activity in a dose-dependent manner. The alkaloids were tested in the same concentration range as the residues and fractions. High inhibitory action upon CP activation showed **2** and **3**, while **1** was less active. The AP activation was strongly suppressed by **2** and nonsignificantly affected by **1** and **3**.

Since the complement system underlies the inflammatory reactions it is expected that the inhibition of complement activity would prevent the development of the inflammatory response. The present study on *L. laxiflora* supposed such correlation. It may be concluded that the residues and nonalkaloid fractions selectively inhibited CP activity, while the alkaloid fractions and **2** were active in both assays. The alkaloids **1** and **3** were moderately active but their inhibitory effect was selective, pointed on CP activity. The results from the application of the residues in two edema models revealed their *in vivo* action. The high suppressive effect upon swelling indicates that pure substances with promising antiinflammatory properties might be further isolated.

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