

Complement Inhibiting Properties of Dragon's Blood from *Croton draco*

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The latex of *Croton draco*, its extracts and several latex components have been investigated for their influence on both classical (CP) and alternative (AP) activation pathways of the complement system using a hemolytic assay. The best inhibition was found for the classical pathway. The latex, ethyl acetate and ethyl ether extracts exhibited extremely high inhibition on the CP (94, 90 and 77%, respectively) at a concentration of 1 mg/ml. The flavonoid myricitrin, the alkaloid taspine and the cyclopeptides P1 and P2 showed high inhibition on CP (83, 91, 78 and 63%, respectively) at a concentration of 0.9 mM.

Key words: *Croton draco*, Components, Classical and Alternative Pathways Inhibition

Introduction

The complement system is an important effector mechanism of the innate (via AP), as well as the acquired (via CP) immunity. Important products as anaphylatoxins, opsonins and chemotactic peptides are generated during the process of complement activation aiming at the development of an inflammatory response to the spread of an infection. As it is well known, different higher plants can inhibit the complement activation (Lasure *et al.*, 1994a). Such plants with anti-inflammatory activity are considered good source for down-regulators of the complement system (Labadie *et al.*, 1989).

Dragon's blood, which is the popular name of the red blood-like latex produced by several *Croton* species including *C. draco*, has been used in the South American countries as a folk remedy for treatment of wounds, inflammation and infections (Ubilas *et al.*, 1994). Biological studies on Dragon's blood, derived from *C. lechleri*, reveal its antibacterial, antitumor, wound-healing and antiviral properties (Chen *et al.*, 1994). *C. panamensis* latex and its extracts of different polarities display a considerable inhibition of the growth of *Bacillus subtilis* and *Pseudomonas fluorescens* (Kostova *et al.*, 1999). So, it was likely that some of the com-

ponents of the latex might have complement-modulating properties and might contribute to its therapeutic and anti-inflammatory effects.

This prompted us to continue our investigations on *C. panamensis* and to study the complement inhibiting (anti-inflammatory) activity of the latex, its ethyl ether, ethyl acetate, butanol and water extracts as well as of several latex components. Later, when these investigations were still going on, systematic revision on the genus *Croton* determined *C. panamensis* as a synonym of *C. draco* (Burger and Huft, 1995).

Experimental

Plant material

The present investigations were initially started with latex from *Croton panamensis* (Klotzsch Muell. Arg.), Euphorbiaceae, collected from the region of Heredia, Costa Rica and authenticated by Dr. H. Navas, National University, Heredia, Costa Rica. In the meantime the taxonomy and systematic of the subgroup of the *Croton* genus producing Dragon's blood have been inspected and *C. panamensis* has been determined as a synonym of *C. draco* Cham. & Sclth. (Burger and Huft, 1995). The latex (10 ml) was freeze-dried to

give a solid material (2.35 g), which was used in hemolytic assays.

General

^1H and ^{13}C NMR spectra were obtained on a Bruker MW 250 spectrometer in CD_3OD or CDCl_3 with TMS as internal standard. EIMS (70 eV): Varian MAT. RP HPLC – analytical: Spherisorb S5 ODS 2 column (250×4.6 mm), flow rate 1 ml/min, detection at 230 nm; preparative: Lichrospher 100 RP 18 column (250×25 mm), flow rate 1.5 ml/min. RLCC (Rotary Locular Counter-current Chromatography): EYELA RLCC UP instrument, flow rate 0.6 ml/min. TLC and preparative TLC: Aluminium sheets, silica gel 60 F_{254} (Merck), detection under UV light or by spraying with Dragendorff reagent.

Chemical studies on latex

Extraction

The latex (500 ml) was mixed with water (500 ml) and extracted in succession with ethyl ether (4×500 ml), ethyl acetate (3×660 ml) and *n*-butanol (5×250 ml) to obtain the ethyl ether (EE; 0.63 g), ethyl acetate (EAE; 5.40 g) and butanol (BE; 13.20 g) extracts. The residual aqueous solution of the latex was freeze-dried to give the water extract (WE; 79.3 g). The chemical composition of the EE extract has been already reported (Kostova *et al.*, 1999).

Isolation of catechins and flavonoids from the EAE

The EAE (1 g) was subjected to RLCC using the lower layer of the solvent system $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ (7:13:9 v/v/v) as a mobile phase to obtain fractions F1–F6. From F2 (315 mg) after RP HPLC ($\text{MeCN}/\text{H}_2\text{O}$, 1:9 v/v) compounds (+)-catechin (**1**, 4.0 mg), (–)-epicatechin (**2**, 6.0 mg), (+)-gallocatechin (**3**, 3.1 mg) and (–)-epigallocatechin (**4**, 5.0 mg) were isolated. F4 after RP HPLC separation ($\text{MeOH}/\text{H}_2\text{O}$, 1:1 v/v) afforded quercitrin (**5**, 10.0 mg), while from F5 (50 mg) quercitrin (**5**, 13.0 mg) and myricitrin (**6**, 4.2 mg) were separated. The structures of these compounds were deduced on the basis of their ^1H and ^{13}C NMR spectra and direct comparison with authentic samples.

Isolation of cyclic peptides from the BE

The above-described extractions of the latex with diethyl ether, ethyl acetate and *n*-butanol proceeded with a formation of stable foam layers between the organic and aqueous phases. Bioassay-guided fractionation of the foam obtained during the butanol extraction step using the hemolysis test (Wagner *et al.*, 1983) led to some active fractions. One of them was worked-up by appropriate chromatographic techniques to give the active cyclic peptides P1 (**7**, $[\text{M}^+]$, 783) and P2 (**8**, $[\text{M}^+]$, 817). Details on their isolation and structure elucidation has already been reported (Rostan *et al.*, 1998).

Isolation of the alkaloid taspine (**9**) from the WE

The water-soluble part (300 ml) left over after the extraction of the latex with ethyl ether, ethyl acetate and *n*-butanol was treated with conc. NH_3 (pH 9.0) and exhaustively extracted with CH_2Cl_2 (4×100 ml). The yellow powder (15.0 mg) obtained after evaporation of the solvent was purified by preparative TLC ($\text{Et}_2\text{NH}/\text{CH}_2\text{Cl}_2$, 1:15 v/v) and crystallized in acetone/ CH_2Cl_2 to yield pure **9** (7.0 mg). The structure was confirmed by its ^1H NMR spectrum and comparison with literature data (Bettolo and Scarpati, 1979).

Microtitre hemolytic complement assay

The hemolytic complement assay was performed in U-welled microtitre dishes following Klerx *et al.* (1983). Since most organic solvents affect human complement activity, all samples with the exception of the water extract (WE) were freed from organic residues by evaporation under nitrogen (below 40 °C) and dried by freeze-drying. The dry material in all samples was dissolved in 10 ml DMSO and diluted with veronal buffered saline, pH 7.3, containing 0.15 mM Ca^{2+} for determination of CP activity and with EGTA-veronal buffer, containing 5 mM Mg^{2+} and 8 mM ethyleneglycol-bis(2-aminoethyl) tetraacetic acid for determination of AP, respectively. Preliminary titration of sera was performed in order to determine the dilution producing 50% haemolysis of target erythrocytes. In the CP assay, sensitised sheep red blood cells at a concentration of 2×10^8 cell/ml were lysed with 1:70 diluted guinea pig serum. In the AP assay unsensitized rabbit erythrocytes at a concentration of 1×10^8 cell/ml were lysed with 1:4 diluted NHS. For measurement of the inhibitory

effect, a mixture of diluted sera and extracts or fractions at various concentrations were preincubated for 30 min at 37 °C and then target cells were added. The plates were incubated again at 37 °C for 60 min (CP) or 30 min (AP). After the final incubation step, the plates were centrifuged to precipitate intact cells and cell-ghosts. To quantify haemolysis, 50 ml of the supernatant were mixed with 200 ml water in 96-well flat-bottom microtiter plates and the absorbance at 405 nm was measured by an ELISA reader (Dynatech, Denkendorf, Germany). Each assay was carried out in triplicate.

Results and Discussion

The complement modulating activity (CP or AP) of the latex and its extracts was examined. The results indicated that the crude latex, EE, EAE and BE inhibited the CP in a dose-dependent manner (Fig. 1a). The latex and EAE had almost an equal and very high inhibitory effect on the CP (94 and 90%, respectively) at a concentration of 1.0 mg/ml. At the same concentration EE and BE also strongly decreased the CP activity (77 and 63%). The amount of the extracts corresponding to 50% inhibition of haemolysis (IC_{50}) was calculated in mg/ml (Table I).

The inhibitory effects of latex, BE, EAE and EE on the AP at the same concentration were lower than those on the CP (Fig. 1b). The influence of WE on the CP and AP was less significant

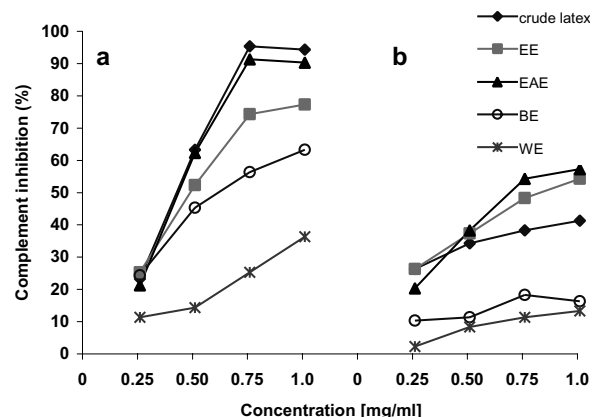


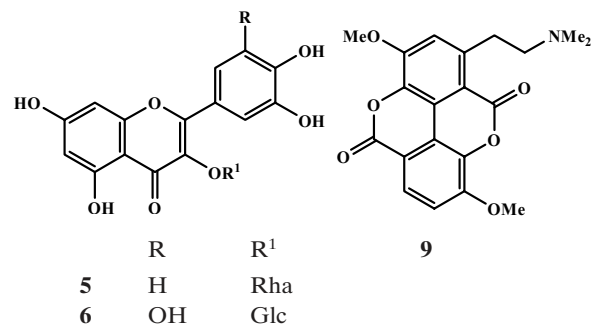
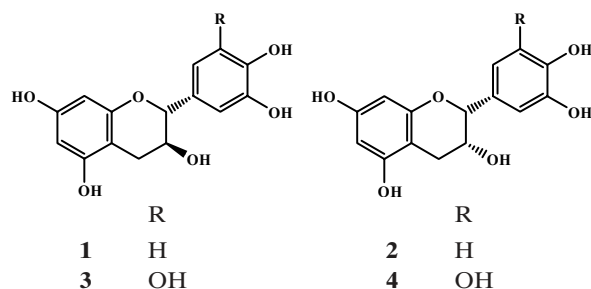
Fig. 1. Complement inhibition by latex of *C. draco* and its extracts on the a) CP and b) AP. EE, ethyl ether extract; EAE, ethyl acetate extract; BE, butanol extract; WE, water extract.

Table I. CP and AP inhibition (IC_{50}) by latex and its extracts.

Extracts	IC_{50} [mg/ml]	
	CP	AP
Crude latex	0.43	–
EAE (ethyl acetate extract)	0.43	0.68
EE (ethyl ether extract)	0.49	0.93
BE (butanol extract)	0.59	–
WE (water extract)	–	–

(up to 36%). No correlation between activities towards the CP and AP was found.

Thus, it was concluded that the latex and its crude extract contained complement-inhibiting compounds of great potency. In order to find the active constituents with complement inhibitory activities, these extracts were worked up by a combination of chromatographic techniques and several constituents were isolated.



The compounds (+)-catechin (1), (–)-epicatechin (2), (+)-gallocatechin (3), (–)-epigallocatechin (4), quercitrin (5) and myricitrin (6) have been previously isolated from the EE of the same latex together with phloroglucinol (10), 4-(2-hydroxyethyl)-benzoic acid (11), 2,5-dihydroxyphenethyl alcohol (12) (Kostova *et al.*, 1999). The preparation of the acetate (13) of 2,5-dihydroxyphen-

ethyl alcohol (**12**) has been described in the same study. The presence of **1–6** was now established in the EAE as well.

The inhibitory activities of the selected EE components are presented in Fig. 2. In terms of the CP inhibition phloroglucinol (**10**) reached up to 78%, followed by 4-(2-hydroxyethyl)-benzoic acid (**11**, 67%). In terms of the AP inhibition only **11** showed 55% inhibition and the inhibitory activities of the remaining compounds were less than 50%.

Further, myricitrin (**6**), epigallocatechin (**4**) and catechin (**1**) from the EAE were analysed (Fig. 3). The CP inhibition exhibited by myricitrin (**6**, 83%) and by epigallocatechin (**4**, 67%) was extremely high. These three compounds inhibited the AP less than 25%. Other components of this extract like quercitrin (**5**), gallicatechin (**3**) and epicatechin

(**2**) are also known to have complement inhibiting properties (Lasure *et al.*, 1994b). According to these authors quercitrin (**5**, 56.2%) is one of the most potent inhibitors of the CP in a series of 54 flavonoids, while epicatechin (**2**) and catechin (**1**) possess a moderate activity (34.3% and 41.6% inhibition of the CP). The same authors observed that the CP inhibition by flavonoids increases with increasing the numbers of hydroxyl groups in ring B. Our study supports this observation. We found that CP inhibition caused by myricitrin (**6**) was 83%, which is higher than that reported by Lasure *et al.* (1994b) for quercitrin (**5**) (56.2%). Compounds **1–4** are present in high amounts in the EAE and strongly contribute to the high immunomodulatory activity of this extract.

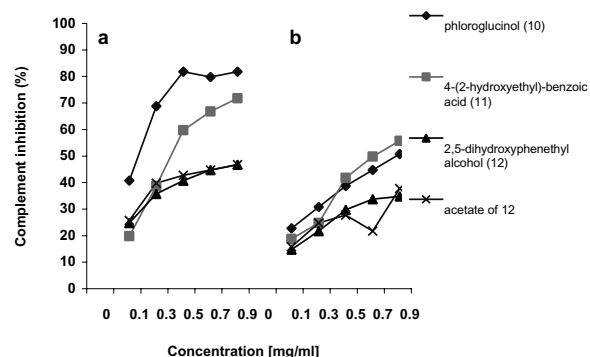


Fig. 2. Complement inhibition by EE extract components on the a) CP and b) AP.

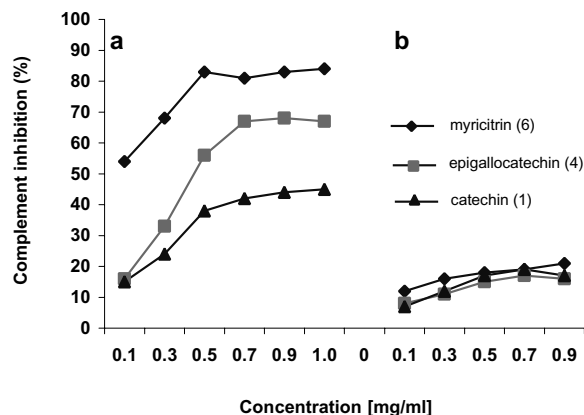
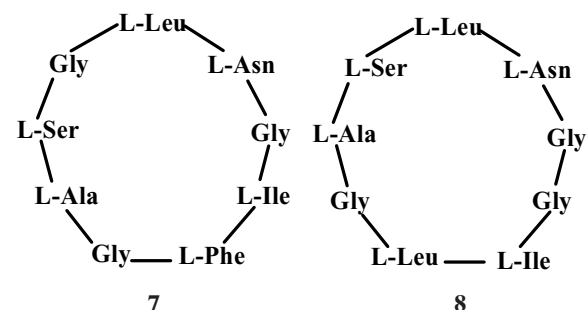


Fig. 3. Complement inhibition by EAE extract components on the a) CP and b) AP.



The inhibitory activity of the two cyclopeptides P1 (**7**) and P2 (**8**), isolated from the BE, was very high, too. The CP inhibition caused by **7** and **8** reached 78 and 63%, respectively (Fig. 4). These

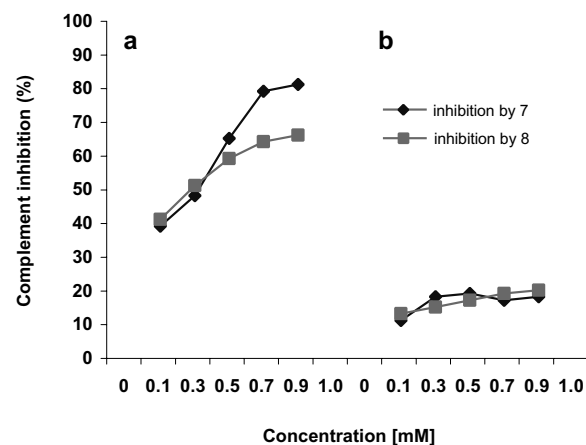


Fig. 4. Complement inhibition by cyclic peptides **7** and **8** on the a) CP and b) AP.

results are in a good agreement with previous reports on the potent immunomodulatory activity of cyclic peptides in the latex of *Jatropha multifida* (Euphorbiaceae) (Kosasi *et al.*, 1989).

The water-soluble extract, the main part of the latex, did not show significant inhibitory activity. However, the alkaloid taspine (**9**), isolated as a minor component of this extract, was very active (Fig. 5). The CP inhibition, displayed by this alkaloid (91%), was the highest observed for all compounds tested in this study. It was reported by Vaisberg *et al.* (1989), that the alkaloid taspine was responsible for the wound-healing properties of Dragon's blood obtained from *C. draconoides* and *C. lechleri*. The presence of taspine in *C. draco* latex could explain its traditional use for wound treatment. There are contradictory data about the cytotoxicity, stimulation of cell migration and tumorigenesis caused by taspine (Itokawa *et al.*, 1991). This observation leads to the conclusion that the appearance of different harmful side effects during treatment with Dragon's blood may be due to the presence of taspine. This allows us to

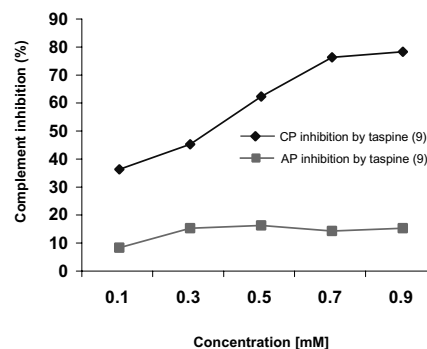


Fig. 5. Complement inhibition by taspine (**9**), isolated from WE on the CP and AP.

recommend the internal application of the taspine-free extracts instead of the whole latex.

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