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Immunomodulatory Activity and Chemical Characterisation of Sangre de Drago (Dragon's Blood) from *Croton lechleri*

Abstract

The immunomodulatory activity of the latex from *Croton lechleri* (sangre de drago) was determined by *in vitro* assays. Classical (CP) and alternative (AP) complement pathways activities were determined in human serum. Intracellular generation of reactive oxygen species (ROS) by human polymorphonuclear leukocytes (PMNs) and monocytes, and phagocytosis of opsonised fluorescent microspheres were measured by flow cytometry. Free radical scavenging activity was evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH). Activity on proliferation of murine lymphocytes was also investigated. In addition, anti-inflammatory activity was assayed *in vivo* by carrageenan-induced rat paw oedema test. Some of the activities were compared with those of the isolated alkaloid taspine. Sangre de drago from *Croton lechleri* showed immunomodulatory activity. It exhibited a potent inhibitory activity on CP and AP of complement system and inhibited the proliferation of activated T-cells. The latex showed free radical scavenging capacity. Depending on the concentration, it showed antioxidant or prooxidant properties, and stimulated or inhibited the phagocytosis. Moreover, the latex has strong anti-inflammatory activity when administered *i.p.* Taspine cannot be considered the main responsible for these activities, and other constituents, probably proanthocyanidins, should be also involved.

Key words

Croton lechleri · Euphorbiaceae · dragon's blood · sangre de drago · anti-inflammatory activity · complement system · oxygen reactive species · phagocytosis · flow cytometry

Abbreviations

AP:	alternative pathway
Con A:	concanavalin A
CP:	classical pathway
DCFH-DA:	2′,7′-dichlorofluorescin diacetate
DPPH:	1,1-diphenyl-2-picrylhydrazyl
FSC:	forward angle light scatter
HPLC:	high-performance liquid chromatography
LB:	lymphoid leukaemia
LPS:	lipopolysaccharide
NMR:	nuclear magnetic resonance
PC:	phagocytic capacity
PI:	phagocytic index
PKC:	protein kinase C
PMA:	phorbol-12-myristate-13-acetate
PMNs:	polymorphonuclear leukocytes
ROS:	reactive oxygen species
SD:	standard desviation
SP:	estimulation of phagocytosis
SSC:	side light scatter
TLC:	thin layer chromatography
UV:	ultraviolet

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Introduction

Sangre de drago is the blood red latex obtained from the bark of several species of *Croton* (Euphorbiaceae), mainly *C. lechleri* Muell. Arg. It is used in traditional medicine in South America and has shown wound-healing activity in cutaneous disorders and, orally, in a dilute form to facilitate the healing of gastric ulcer, reducing ulcer size and bacterial content of the ulcer. Moreover, the latex showed anti-inflammatory, antibacterial, antiviral and antioxidant-prooxidant activities [1], [2], [3], [4], [5], [6], [7].

Several compounds have been isolated from sangre de drago. Catechin, epicatechin, gallocatechin, epigallocatechin (monomeric flavan-3-ols) and proanthocyanidins of several molecular sizes were its major constituents [8]. Among them, SP-303, a large proanthocyanidin oligomer [1] with antiviral activity [9], is under clinical development for the oral treatment of diarrhoea and topical treatment of genital and anal herpes simplex lesions in patients with AIDS [10]. As minor constituents, taspine (alkaloid), 3′,4-*O*-dimethylcedrusin (dihydrobenzofuran lignan), and several diterpenes were identified [2], [4].

In order to have a best definition of the tested product, prior to the activity testing, sangre de drago samples were characterised using different physico-chemical, chromatographic and spectroscopic methods. To evaluate the immunomodulatory activity several *in vitro* assays were performed and anti-inflammatory activity was assayed *in vivo*.

Materials and Methods

Plant material

Sangre de drago was collected by bark incision from *Croton lechleri* Müell. Arg., from the province of Napo (Ecuador). A voucher specimen of the plant (BCF48066) has been included in the BCF Herbarium (Faculty of Pharmacy, University of Barcelona). The latex was freeze-dried for conservation.

Chemicals

Hydrogen peroxide was obtained from Jansen (Geel, Belgium), 2′,7′-dichlorofluorescin diacetate from Serva (Heidelberg, Germany), sodium tungstate and pyrogallol from Merck (Darmstadt, Germany), [^3H]thymidine from NEN Product (Boston, MA, USA), hide powder from European Pharmacopeia (Strasbourg, France) and fluorescence-labelled spheres (Fluospheres®, 2 μm diameter, from Molecular Probes Europe BV). HPLC standards: (+)-catechin, (−)-epicatechin, (−)-epigallocatechin and procyanidin B1 and B2 were purchased from Extrasynthèse (Genay, France). Others chemicals were obtained from Sigma Chemical Co (St. Louis, MO, USA).Taspine was isolated in our laboratory from the latex of *Croton lechleri* as described previously [4] and was identified by ^{1}H-NMR, ^{13}C-NMR and mass spectral data.

Characterisation of latex samples

Before lyophilisation, pH and density of the liquid samples were measured. Dry residue by lyophilisation was also determined. The presence of taspine was investigated by TLC, using silica gel 60 F_{254} plates. Lyophilised latex was dissolved in MeOH and taspine was used as reference. The plate was eluted with CH_2Cl_2 : MeOH (13 : 7) and detection was performed with iodoplatinate reagent, examining in daylight. The HPLC fingerprint of sangre de drago was carried out on a Hewlett Packard HP1050 series chromatograph using a Teknokroma Spherisorb (C18) ODS2 column (250 mm × 4.6 mm, 5 μm particle size). The mobile phases were: 2% aqueous acetic acid and acetronitrile. The elution (flow rate: 1 mL/min, room temperature) was as follows: gradient from 0.1 to 15% CH_3CN in 15 min; isocratic for 5 min; gradient up to 20% CH_3CN in 5 min; gradient up to 30% CH_3CN in 5 min. Detection was performed at 280 nm using a UV detector.

The ^{13}C-NMR spectrum was recorded in DMSO at 300 K in a Varian Unity 200 instrument (200 MHz).

The polyphenol profile was investigated by TLC using silica gel plates. Lyophilised latex was dissolved in MeOH. The plate was eluted with AcOEt : HCOOH:AcOH : H_2O (100 : 11 : 11 : 27) and detection was performed with Berlin blue reagent [aqueous solutions of $FeCl_3$ (1%) and $K_3Fe(CN)_6$ (1%) in proportion 1 : 1], examining in daylight. The presence of dark blue spots is indicative of tannins containing *ortho*-trihydroxy groups.

The tannin content of sangre de drago was measured using a method adapted from that described for *Hamamelis* leaf in the 3rd Edition of the European Pharmacopeia [11]. Results are expressed as pyrogallol.

Haemolytic assay for human complement activity

Classical (CP) and alternative (AP) complement pathway activities were determined in human pooled serum [12], used as source of complement. The amount of haemoglobin released was measured at 405 nm.

Assay for DPPH free radical scavenging activity

Free radical scavenging activity was evaluated according to Malencic et al. [13] using 1,1-diphenyl-2-picrylhydrazyl (DPPH). The absorbance was determined at 515 nm.

ROS measurements by flow cytometry

Changes in intracellular ROS levels in human leukocytes were determined by flow cytometry as described previously [14]. ROS production was measured using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as fluorescence probe. Cellular fluorescence is proportional to ROS production. H_2O_2 100 μM or PMA (phorbol myristate acetate) 10 μM were used as stimulant of ROS production. Viability was measured by propidium iodide exclusion. Statistical analysis was done with one-way analysis of variance (ANOVA) and Student's t test. The production of ROS was analysed in neutrophils, but also in monocytes (lower producers of ROS) and lymphocytes (which are able to generate small amounts of ROS when unseparated leukocytes are activated by PMA).

Phagocytosis evaluation by flow cytometry

Phagocytosis was evaluated using opsonised (with 10% human serum) fluorescent spheres according to Steinkamp et al. [15] with modifications [16]. The fluorescence of extracellular spheres was quenched by adding trypan blue. Viability was measured by propidium iodide exclusion. The following parameters were calculated: phagocytic capacity (PC), which was expressed as the percentage of cells that ingested one or more

particles; stimulation of phagocytosis (SP), expressed as the percentage of variation of PC; and phagocytic index (PI), which was defined as the average number of ingested particles per cell and was calculated from the fluorescence distributions (Fig. **1**) [16]. Statistical analysis was done with one-way analysis of variance (ANOVA) and Student's t test.

Different assays in which phagocytosis was evaluated in human leukocytes, monocytes from a human mononuclear suspension and in monocytes/macrophages from a murine peritoneal exudate were performed. Activity of monocytes is much better evaluated after the cells are separated from neutrophils, due to an overlapping between neutrophils and phagocytic monocytes (ingestion induces a strong scatter signal).

T-cell proliferation assays: [^3H]thymidine uptake by normal and tumoural murine lymphocytes

The test was performed according to Fernández et al. [17]. All cultures were carried out in 96-well plates. Radioactivity incorporated ([^3H]thymidine) into cells was measured by means of a liquid scintillation beta counter. Growth percentage for each treatment was calculated as % growth = 100×(cpm exp. sample – cpm control)/cpm control. Viability was evaluated by the trypan blue exclusion test. Statistical analysis was done with one-way analysis of variance (ANOVA) and Dunnet's multiple test.

Carrageenan-induced paw oedema test in rat

The carrageenan-induced oedema test in rat hind paw was described earlier [18]. Statistical analyses of experimental data were determined by analysis of variance (ANOVA) and Scheffé's test.

Results

Characterisation of latex samples

The pH of sangre de drago of *Croton lechleri* used in the present work was 3.9, the density was 1.1 g/mL and the dry residue 26%. These data are in accordance with the records for this product in our laboratory (pH 3.8 to 3.9, density 1.07 – 1.1 g/mL, dry residue 23 – 26%). The presence of taspine was confirmed by TLC, appearing as a zone with the same Rf (0.21) and colour as the reference. The fingerprint by ^{13}C-NMR is shown in Fig. **2**. The content of tannins was 18% with reference to the liquid latex (69.2% with reference to dry residue). The TLC profile for polyphenols showed several blue zones at the following Rf values: 0.91, 0.83, 0.69, 0.60 and 0.48 (faint zone). The fingerprint by HPLC is shown in Fig. **3**.

Human complement activity

Sangre de drago from *C. lechleri* exhibited potent inhibitory activity towards the CP and AP of human complement system with an IC_{50} = 5 μg/mL and 185 μg/mL, respectively (Fig. **4**). However, the

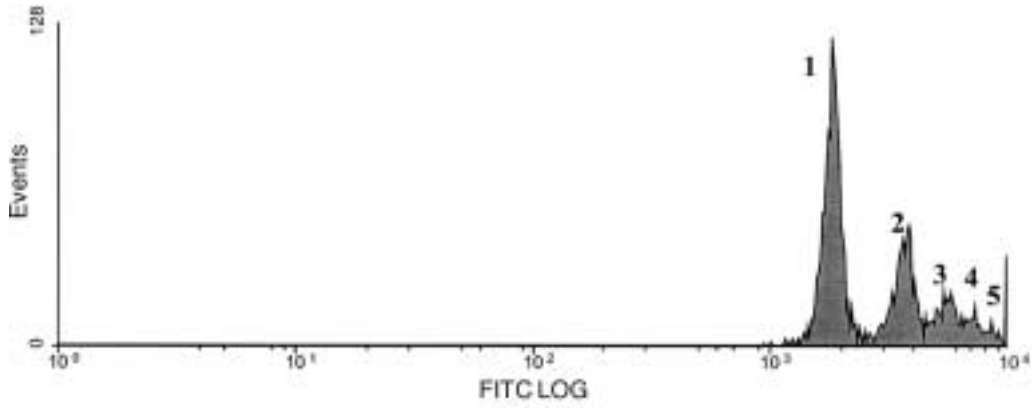

Fig. **1** Single-parameter histogram of fluorescence distribution of cell-phagocytised spheres obtained by displaying only fluorescence signals associated with light scatter signals from cells. Fluorescence was plotted on a $\log_{10}$ scale from channel numbers 0.1 to 1023 on the abscissa. Values at the ordinate correspond to the number of phagocytes at each channel. Fluorescence peaks according to the number of particles internalised by cells are observed.

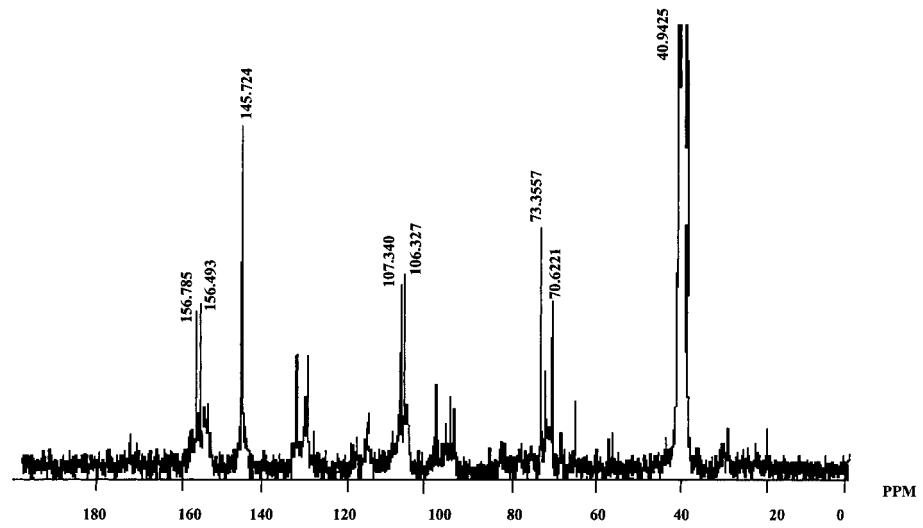

Fig. **2** ^{13}C-NMR spectrum of sangre de drago from *C. lechleri* in DMSO (200 MHz, 300 K).

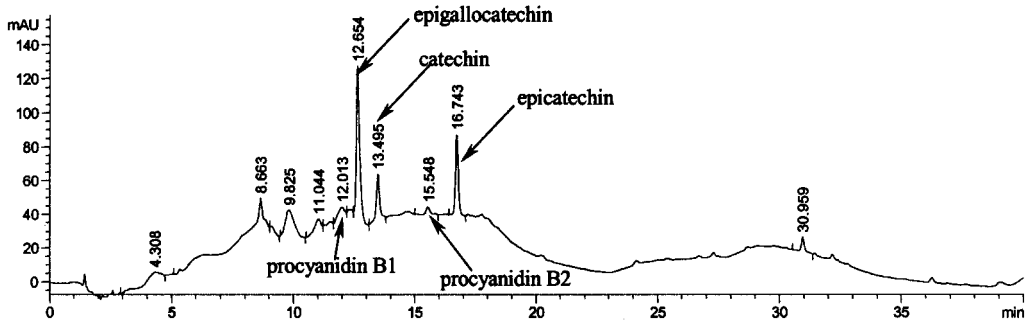

Fig. **3** HPLC chromatogram of sangre de drago from *Croton lechleri*. The retention time (R_t, min) of the standards were: (+)-catechin,13.5; (–)-epicatechin, 17; (–)-epigallocatechin, 12.; procyanidin B1, 12 and procyanidin B2, 15.

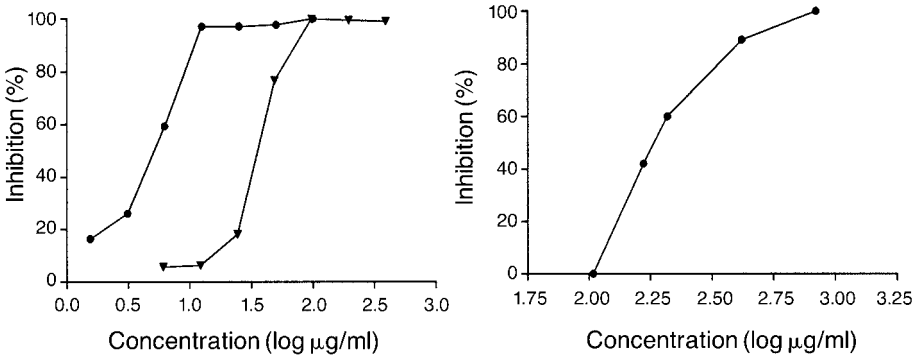

Fig. **4** The effect as percentages of inhibition of sangre de drago from *C. lechleri* (●) and taspine (▼) on CP (left) and AP (right) complement activity (N = 4).

effect of latex was higher than that of taspine which showed an inhibition of the CP with an IC_{50} = 38 μg/mL and no effect on the AP, even at a concentration of 675 μg/mL. Quercetin, used as the reference control, showed an IC_{50} = 33.7 μg/mL on the CP.

DPPH free radical scavenging activity

Sangre de drago from *C. lechleri* decreased the concentration of DPPH radical (IC_{50} = 7.73 μg/mL); this activity was similar to that of quercetin (reference control), catechin and epicatechin (Table **1**).

ROS measurements by flow cytometry

Preliminary experiments were performed to detect the influence of sangre de drago in the viability and morphology of not stimulated cells. At concentrations between 10 μg/mL and 100 μg/mL, and according to IP exclusion, no significant differences in the total number of viable cells were observed between treated and control samples (Table **2**). However, the latex increased the percentage of damaged cells, at concentrations of 50 μg/mL or higher, detected by an FSC/SSC increase on neutrophils and monocytes (Fig. **5**). In addition, at concentrations higher than 25 μg/mL, the latex produces an activation of ROS generation in human neutrophils (Fig. **6**).

For the measurements of ROS production after stimulation of the cells with H_2O_2 or PMA, quercetin was used as the reference control. The results obtained in human leukocytes treated with sangre de drago from *C. lechleri*, after stimulation of the cells with H_2O_2 or PMA, are reported in Table **3** and Fig. **7**. Sangre de drago caused an inhibition of ROS production in human neutrophils at concentrations between 0.01 μg/mL and 1 μg/mL upon stimulation with H_2O_2. When cells were stimulated by PMA, an inhibition was also observed at concentrations from 0.0001 μg/mL to 1 μg/mL. However, at concentrations ranging from 30 μg/mL to 50 μg/mL, an increase of ROS production was observed in human neutrophils stimulated with H_2O_2 or PMA. In human monocytes, stimulated by H_2O_2, sangre de drago showed inhibition at concentrations ranging from 0.01 μg/mL to 1 μg/mL. When the stimulation was done with PMA, the inhibition was observed at 0.0001 μg/mL to 10 μg/mL. Inhibition was also detected in the same range of concentration in lymphocytes stimulated with PMA.

Phagocytosis evaluation

In the phagocytosis evaluation, lipopolysaccharide (LPS) from *Escherichia coli* was used to tune the method, since it is known that bacterial LPS enhances phagocytic activity [19]. Concentration-dependent modulation of the phagocytosis of human leukocytes was observed for sangre de drago from *C. lechleri*. The latex exerted a concentration-dependent enhancement of the phagocytosis in human neutrophils and monocytes at concentrations of 5 μg/mL and 10 μg/mL (Table **4**). When mononuclear cells were isolated and separated from neutrophils, the stimulation of phagocytosis observed in human monocytes was more pronounced. Positive SP was correlated with the increase of PI. At about 30 min incubation time, the monocytes seem to have a

Table **1** Scavenging effects of sangre de drago and different standards on the DPPH radical

Treatment	*IC_{50} (μg/mL) ± SD*
Sangre de drago	7.73 ± 1.87
Epicatechin	4.29 ± 0.50
Catechin	5.08 ± 0.25
Quercetin	5.29 ± 0.43

Data expressed as mean ± SD (N = 4).

Table **2** ROS measurements in non-stimulated human neutrophils and monocytes treated with sangre de drago from *Croton lechleri*

	Human neutrophils		*Human monocytes*	
Treatment (μg/mL)	***MCF***	***MCFt-MCFc***	***MCF***	***MCFt-MCFc***
0	5.16 ± 0.09		9.79 ± 0.27	
10	10.12 ± 0.28	4.96	11.44 ± 0.19	1.51
25	16.75 ± 0.02	11.59	12.51 ± 0.21	2.72
50	30.13 ± 1.46	24.97**	12.16 ± 0.56	2.37
75	26.11 ± 1.22	20.95**	9.63 ± 0.12	–0.16
100	28.25 ± 1.28	23.09**	9.60 ± 0.05	–0.19

MCF: Mean channel of fluorescence.
Data are expressed as mean ± SD (N = 6). ** $p < 0.01$.
The differences in MCF of untreated (MCFc) and treated (MCFt) samples show a stimulant effect of sangre de drago on ROS generation in neutrophils at concentrations of 50 μg/mL or higher.

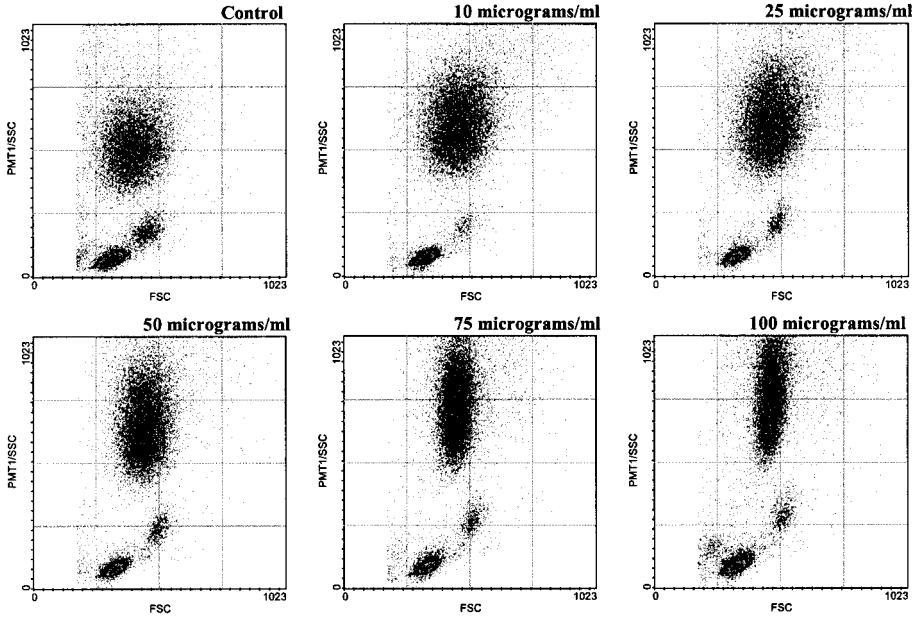

Fig. **5** Cytograms (FSC × SSC) obtained from a suspension of human leukocytes treated with sangre de drago from *Croton lechleri* (5 min at 37 °C). Concentrations of sangre de drago higher than 50 μg/mL induce a pronounced change in FSC and SSC of human neutrophils, in comparison with untreated cells (control cells), which is detected as an up- and right-displacement of the cells. At least 20 000 living neutrophils were analysed for each sample. A representative experiment is shown (N = 4).

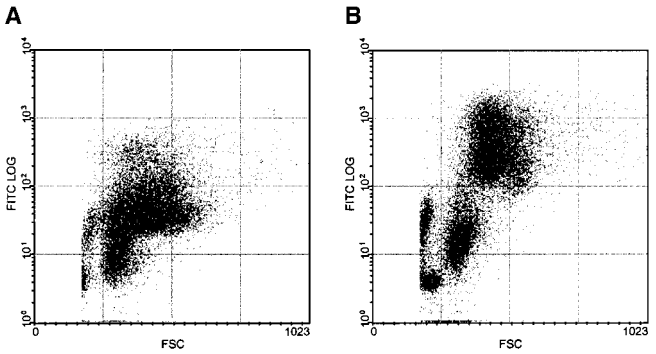

Fig. **6** Cytograms (FITC-LOG × FSC) obtained for ROS measurements in unstimulated human leukocytes untreated (**a**) or treated (**b**) with 50 μg/mL of sangre de drago from *Croton lechleri*. An up-displacement of the cells is observed, indicating a stimulating action of sangre de drago on ROS production. At least 20 000 living neutrophils were analysed for each sample A representative experiment is shown (N = 6).

higher PI than neutrophils. At 50 μg/mL, an inhibition of phagocytosis in human monocytes was observed, with a decrease of SP and PI. In murine peritoneal monocytes/macrophages, a concentration-dependent stimulation of phagocytosis was recognized (Table **3**). Taspine produced more stimulation of phagocytosis than sangre de drago in human monocytes. Nevertheless, no effect was observed on human PMN and murine peritoneal monocytes/macrophages.

T-cell proliferation assays

Sangre de drago from *C. lechleri* did not exert a significant effect on normal splenocytes proliferation, but inhibited mitogen-stimulated with Con A splenocytes and lymphoid leukaemia cells growth (Table **5**).

Carrageenan-induced paw oedema

The time-dependent anti-inflammatory activity of sangre de drago from *C. lechleri* by *i.p.* administration is shown in Fig. **8**. The doses of 50 mg/kg and 25 mg/kg exhibited a significant inhibition of 100 % (Table **6**), however, a peritoneal exudate was observed, the effect being probably due to counter irritant activity. This exudate was not observed at doses lower than 25 mg/kg. The anti-inflammatory effect of sangre de drago at 5 mg/kg *i.p.* was comparable to that of 20 mg/kg *i.p.* of naproxen during the first 4 h.

Table **3** Effect of sangre de drago from *Croton lechleri* on ROS production in human leukocytes

Treatment (μg/mL)	Neutrophils		Monocytes		Lymphocytes	
	MCF	% INH	MCF	% INH	MCF	% INH
Stimulation by H_2O_2						
Control	11.80 ± 1.12		3.73 ± 0.22		1.27 ± 0.04	
0	42.00 ± 4.04		13.59 ± 2.34		2.84 ± 0.02	
0.001	36.10 ± 3.37	19.5	14.80 ± 0.23	–12.3	2.67 ± 0.01	10.8
0.01	25.90 ± 2.68	53.3**	8.11 ± 0.30	55.6*	1.74 ± 0.14	70.1
0.1	36.80 ± 3.67	17.2*	11.04 ± 0.14	25.9*	2.35 ± 0.30	31.2
1	36.30 ± 3.90	18.9*	10.51 ± 0.17	31.2*	2.10 ± 0.45	47.1
10	41.00 ± 4.49	3.3	13.02 ± 0.16	5.8	2.81 ± 1.01	1.9
20	45.00 ± 5.31	–9.9	13.18 ± 3.2	4.2	2.33 ± 0.91	32.5
30	48.50 ± 5.89	–21.5*	15.53 ± 3.2	–19.7	2.50 ± 0.21	21.7
40	51.10 ± 6.74	–30.1*	15.94 ± 2.9	–23.8*	2.63 ± 1.17	13.4
50	56.00 ± 7.58	–46.4**	16.60 ± 2.8	–30.5*	2.58 ± 1.10	16.6
Stimulation by PMA						
Treatment (μg/mL)	**Neutrophils**		**Monocytes**		**Lymphocytes**	
	MCF	**% INH**	**MCF**	**% INH**	**MCF**	**% INH**
control	6.24 ± 0.27		7.35 ± 0.21		5.38 ± 0.08	
0	32.35 ± 3.05		20.15 ± 2.04		10.57 ± 1.17	
0.0001	15.76 ± 0.32	63.5*	13.02 ± 0.49	55.7*	6.48 ± 1.82	78.8*
0.001	16.23 ± 0.59	61.7*	12.83 ± 0.40	57.2*	6.11 ± 0.12	85.9*
0.01	17.22 ± 0.98	57.9**	13.79 ± 0.45	49.7*	6.37 ± 0.31	80.9*
0.1	18.55 ± 0.49	52.9**	14.06 ± 0.29	47.6*	6.96 ± 0.08	69.6*
1	20.99 ± 0.76	43.5**	16.10 ± 0.60	31.6*	6.89 ± 0.40	70.9*
10	28.13 ± 0.05	16.2	16.91 ± 0.40	25.3*	9.05 ± 0.01	29.3
30	40.52 ± 0.94	–31.3*	16.84 ± 0.81	25.9*	14.20 ± 0.47	–69.9*
50	43.42 ± 0.89	–42.4**	19.25 ± 0.57	7.0	14.14 ± 0.24	–68.9*

Data expressed as mean ± SD (N = 8).
MCF: Mean channel of fluorescence.
Separate measurements of MCF in neutrophils, monocytes and lymphocytes were obtained by combination of FSC × SSC and gating each population in their respective fluorescence histograms. Negative values of inhibition means stimulation.
** $p < 0.01$, * $p < 0.05$.

Discussion

The present study establishes the anticommplementary, antioxidant/prooxidant, phagocytosis modulating and anti-inflammatory activities of the latex from *Croton lechleri* in the models used.

Sangre de drago exhibited potent inhibitory activity towards the CP and AP of human complement. Major constituents of sangre de drago are catechins (monomeric flavan-3-ols) and proanthocyanidins. These compounds are probably responsible for this effect, since the complement-modulating activity of a series of monomeric flavan-3-ols and proanthocyanidins have been reported in the literature [20]. A possible synergistic action between these compounds has been also suggested [20].

In the present study, we chose to use the stable radical DPPH, as an easy and rapid way to evaluate the capacity of sangre de drago to scavenge free radicals independently of any enzymatic activity. Flow cytometric assays were applied to define the effect of sangre de drago on ROS production in human leukocytes. In ROS measurements by flow cytometry, cells were treated with H_2O_2 and PMA to cause increased oxidative metabolism. H_2O_2 is a reactive oxygen intermediate because it can be transformed in ROS in the cell. Activation of cells by PMA, a stimulator of protein kinase C (PKC), leads to a marked increase in the phosphorylation of multiple proteins. As the result, NADPH oxidase is activated, leading to an increase of ROS. Sangre de drago showed free radical scavenging ability by the DPPH test. Moreover, sangre de drago reduced the intracellular ROS formation in human neutrophils at concentrations equal to or lower than 1 μg/mL, and increased this formation at concentrations of 30 μg/mL or higher, when the cells were stimulated with H_2O_2 or PMA. According to the results, the antioxidant activity of sangre de drago is related to its scavenger capacity, but also other mechanisms, perhaps PKC inhibition might be implicated. Taspine is not responsible for this activity, however catechins and proanthocyanidins (the antioxidant properties of which are well known) are probably involved in the modulation of ROS production. Concerning the antioxidant/prooxidant behaviour shown by sangre de drago, it has also been described for several polyphenols, such as quercetin, that can behave as antioxidant or prooxidant depending on the concentration and free radical source.

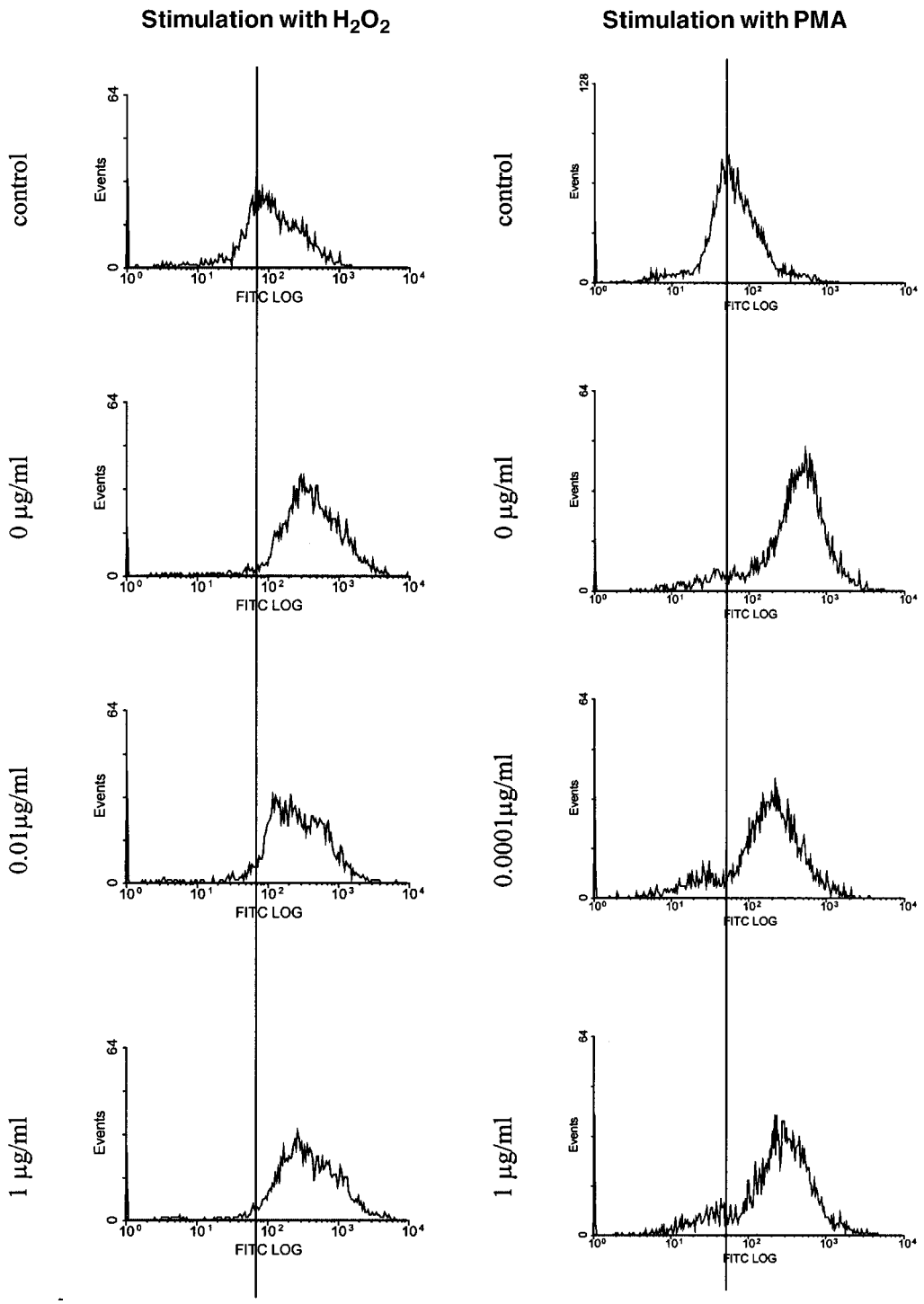

Fig. **7** Histograms of fluorescence distribution showing the effect of sangre de drago from *Croton lechleri* on ROS production in human neutrophils stimulated with H_2O_2 (left panel) and PMA (right panel). Intracellular fluorescence was measured by flow cytometry and is plotted on a $\log_{10}$ scale from channel numbers 0.1 to 1023 on the abscissa. A representative experiment is shown (N = 8).

Besides, other authors [6] have determined the total reactive antioxidant potential of sangre de drago by monitoring the intensity of luminol enhanced chemiluminescence by peroxyl radicals. At concentrations of 1 mg/mL or higher, sangre de drago showed an antioxidant effect, but lower concentrations yielded prooxidant activity. Our results prove that sangre de drago at concentrations of 50 μg/mL or higher caused an increase of the percentage of damaged neutrophils.

Results on phagocytosis evaluation showed that sangre de drago stimulates/inhibits the phagocytosis in human monocytes and PMN depending on the concentration. At concentrations ranging from 5 μg/mL to 20 μg/mL, an enhancement of phagocytosis in human monocytes and in murine macrophage-monocytes was detected by an increase of PC and PI. However, the reduction in phagocytosis in human monocytes occurred mainly at the 50 μg/mL concentration.

Sangre de drago inhibited lymphocyte proliferation mitogen-stimulated with Con A and lymphoid leukaemia cell growth, indicating that the latex inhibits the cell-mediated immune response. Furthermore, the normal splenocytes stimulated with Con A were significantly more sensitive to the latex than lymphoid leukaemia cells. Recently, a dose-dependent antiprolifera-

Table **4** Effect of sangre de drago of *Croton lechleri* on phagocytosis

Treatment (µg/mL)	*In human leukocytes (neutrophils + monocytes)*							
				Phagocytic cells (%) of n *particles*				
	PC ± SD	*SP*	*MCF ± SD*	*1*	*2*	*3*	*≥4*	*PI*
0	12.67 ± 1.90		206.46 ± 2.3	75.89	17.84	3.55	2.55	1.32
0.75	14.96 ± 2.40	18.07	208.60 ± 5.5	74.01	19.06	4.16	2.78	1.36
1.25	13.26 ± 3.34	4.66	207.67 ± 3.0	76.12	17.54	3.88	2.48	1.33
2.5	14.72 ± 3.23	16.18	213.62 ± 5.4	73.76	18.74	4.31	3.18	1.37
5	17.84 ± 3.17	40.81*	216.07 ± 7.3	71.72*	20.01*	4.82*	3.45*	1.40
10	17.90 ± 3.11	41.28*	216.70 ± 9.9	70.96*	20.73*	4.63*	3.67*	1.41
20	13.08 ± 1.77	3.23	204.43 ± 3.4	72.25	17.58	2.91	2.27	1.30
40	9.01 ± 1.15	–28.88	199.65 ± 3.2	81.99	14.59	1.30	1.37	1.21
80	6.65 ± 1.76	–47.51*	196.82 ± 2.4	83.03	14.59	1.48	0.90	1.20
	In human monocytes							
Treatment (µg/mL				*Phagocytic cells (%) of* n *particles*				
	PC ± SD	*SP*	*MCF ± SD*	*1*	*2*	*3*	*4*	*≥5*	*PI*
0	33.37 ± 5.08		231.19 ± 5.12	60.7	4.8	5.6	16.4	12.3	2.13
0.1	26.97 ± 3.95	10.79	221.52 ± 12,14	63.5	5.2	7.1	14.7	9.2	2.00
0.5	35.37 ± 4.32	5.99	226.13 ± 1,20	64.0	3.5	4.6	16.6	11.4	2.08
1	36.34 ± 6.68	8.90	235.89 ± 12,80	58. 6	4.8	6.2	17.4	12.8	2.20
5	45.82 ± 2.55	37.31*	244.19 ± 0,09	56.1	3.8	5.7	19.0	15.4	2.34
10	51.24 ± 3.13	53.55*	259.03 ± 10.0	49.9**	3.9*	7.1	20.7	18.3**	2.53
20	57.32 ± 0.05	71.77**	268.76 ± 8.77	48.0*	3.3*	5.7	21.3	21.7**	2.65
30	40.16 ± 10.8	20.35	231.55 ± 13.42	57.5	5.6	8.3	16.9	11.5	2.19
50	11.09 ± 2 .33	–66.77**	191.69 ± 12.0	78.3**	5.2	4.3	9.0*	3.1**	1.53
	In murine macrophage-monocytes							
0	5.85 ± 1,60		127.20 ± 3.89	79.8	4.8	4.1	8.9	2.3	1.49
1.25	6.06 ± 3.01	3.59	127.60 ± 7.22	75.1	5.0	4.3	8.6	7.0	1.67
2.5	5.86 ± 1.33	0.17	127.50 ± 3.79	75.0	5.5	4.4	9.3	5.9	1.66
5	6.72 ± 0.81	14.87	127.60 ± 6.34	74.3*	5.1	4.7	9.2	6.6*	1.68
10	8.53 ± 0.52	45.81*	128.10 ± 4.73	74.7*	4.9	4.6	9.1	6.7*	1.68
20	11.32 ± 2.33	93.50*	130.30 ” 8.05	72.5*	4.9	4.4	10.5	7.6*	1.76

Data show the phagocytic capacity (PC), stimulation of phagocytosis (SP), mean channel of fluorescence (MCF), percentage of phagocytic cells according to the number of ingested spheres, and phagocytic index (PI).
Data expressed as mean ± SD (N = 6). ** $p < 0.01$, * $p < 0.05$. Negative values of stimulation means inhibition.

Table **5** Effect of sangre de drago from *Croton lechleri* on lymphocyte proliferation

Treatment (µg/mL)	*Cell proliferation inhibition (%)*		
	NS	*NS + Con A*	*LB cells*
1	n. e.	n. e.	n. e.
10	n. e.	43*	30
100	n. e.	93**	76**

NS: normal splenocytes; NS + Con A: normal splenocytes stimulated with Con A; LB: lymphoid leukaemia. n. e. means no effect. N = 5. ** $p < 0.01$. * $p < 0.05$.

tive effect on human leukaemic cells was reported for the latex of *C. lechleri* [21]. 3′,4-*O*-Dimethylcedrusin, a minor constituent of sangre de drago, could be implicated in this activity because the potential antiproliferative and antitumoural activity of this lignan has been previously reported and it is known that lignans exhibit some kinds of antitumour activities [22].

Finally, sangre de drago *i. p.* administered was strongly effective in acute inflammation induced by carrageenan. The anti-inflammatory activity of the latex was not previously reported in the literature. However, significant anti-inflammatory activity in three inflammation models, including carrageenan-induced paw oedema, was described for taspine hydrochloride administered orally [4]. In addition to taspine, catechins and proanthocyanidins are probably involved in the anti-inflammatory activity.

In conclusion, sangre de drago from *C. lechleri* has immunomodulatory and anti-inflammatory activities. Taspine is not the only constituent responsible for these effects; other compounds (catechins and proanthocyanidins) might be also involved.

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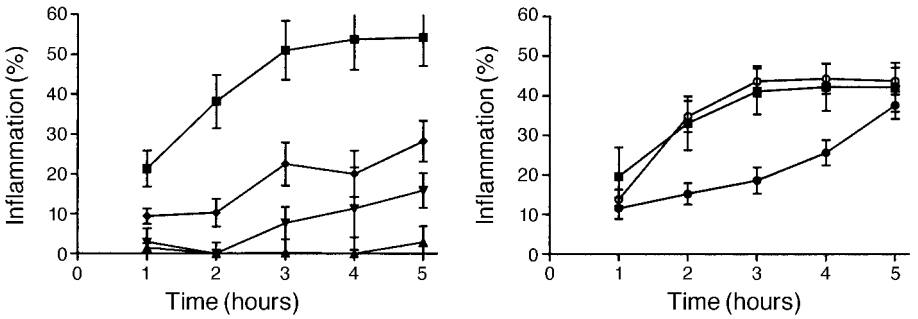

Fig. **8** Time-dependent anti-inflammatory activity of sangre de drago by *i.p.* administration [control (■), 1 mg/kg (○), 5 mg/kg (●), 10 mg/kg (◆), 25 mg/kg (▼) and 50 mg/kg (▲) on oedema in rat paw induced by carrageenan (N = 6). Results were expressed as the increase in paw volume due to carrageenan administration ± S.E.

Table **6** Inhibitory effect of sangre de drago on oedema in rat paw induced by carrageenan

Dose (mg/kg)	*Inflammation inhibition (%)*				
	1 h	*2 h*	*3 h*	*4 h*	*5 h*
50	92.9**	100**	99.5**	100**	94.5**
25	86.6**	100**	85.0**	78.9**	70.7**
10	56.2*	73.1**	55.9**	62.7**	47.9*
5	40.9	53.9	54.6*	39.1*	10.8
1	8.0	12.7	1.9	–0.3	–2.8
Naproxen[a]	0	51.2	43.5*	44.22*	40.9*

Results were obtained by *i.p.* administration of latex (N = 6).

[a] Typical values obtained in one experiment (20 mg/kg).

Statistically significant difference between treated and control groups: * $p < 0.05$; ** $p < 0.01$.

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