Effects of leaf extracts from *Croton zambesicus* Müell. Arg. on hemostasis

Séverine Robert [a],[*],[1], Chiara Baccelli [b],[1], Philippe Devel [a], Jean-Michel Dogné [a], Joëlle Quetin-Leclercq [b]

[a] Department of Pharmacy, Drug Design and Discovery Center, University of Namur, FUNDP, Rue de Bruxelles, 61, 5000 Namur, Belgium
[b] Laboratoire de Pharmacognosie, Louvain Drug Research Institute, Université catholique de Louvain, Avenue E. Mounier, 72, 1200 Bruxelles, Belgium

ABSTRACT

Ethnopharmacological relevance: The leaf decoction of *Croton zambesicus* Müell. Arg. (*Euphorbiaceae*; syn. *Croton amabilis* Müell. Arg., *Croton gratissimus* Burch) is traditionally used in Benin to treat hypertension.
Aim of the study: As hypertension and thromboembolism are often associated in several cardiovascular diseases, we studied the potential effects of leaf extracts from *Croton zambesicus* on hemostasis.
Materials and methods: We prepared the dichloromethane and aqueous extracts from the air-dried leaves of *Croton zambesicus* and separated the aqueous extract in its aqueous and dichloromethane fractions. The potential effects of these four extracts/fractions were investigated on red blood cells integrity using spectrophotometric lysis assays, on primary hemostasis using platelet aggregation studies and on secondary hemostasis using calibrated automated thrombin generation assays and coagulation factors inhibition tests.
Results: In the *in vitro* testing, we found that none of the tested extracts/fractions exhibit hemolytic or antiplatelet activity. However, they display a moderate but significant anticoagulant activity which would be mediated through the direct inhibition of thrombin, FXa and TF/FVIIa. The active anticoagulant compound(s) seem to be mainly in the aqueous extract and especially in its aqueous fraction.
Conclusions: This experimental work reported for the first time the anticoagulant effect of leaf extracts from *Croton zambesicus*. These findings are of particular interest as the leaves from *Croton zambesicus* are commonly used in infusion by local population and may provide a new natural source for the development of original anticoagulant agents. Furthermore, this activity, associated with the vasorelaxant properties of some of its diterpenes may prove to be interesting for the prevention of cardiovascular diseases in traditional medicine.

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1. Introduction

Herbal remedies used in traditional medicine provide an interesting and still largely unexplored source for the development of new drugs (Cos et al., 2006). *Croton zambesicus* Müell. Arg. (*Euphorbiaceae*; syn. *Croton amabilis* Müell. Arg., *Croton gratissimus* Burch) is a tree reaching 10 m in height and is widely spread in tropical Africa. This plant is extensively used in African traditional medicine (Watt and Breyer-Brandwijk, 1962). The leaf decoction is employed in Benin to treat hypertension, urinary infection and fever associated to malaria (Adjanohoun et al., 1989; Adjanohoun and de Souza, 2002) and in Nigeria to treat diarrhea, dysentery and malaria (Ajibesin et al., 2008). The root is used in Nigeria as antidiabetic and antimalarial remedy (Okokon et al., 2005, 2006) and in Sudan for menstrual pain and constipation (el-Hamidi, 1970; Ngadjui et al., 2002).

Few pharmacological studies have been carried out on *Croton zambesicus* biological activities. Antimalarial activity was observed with extracts from the stem bark (Boyom et al., 2009; Okokon and Nwafor, 2009) and from the seeds (Ali et al., 2002). The stem bark also displays antimicrobial activity (Abo et al., 1999). The ethanolic leaf extract possesses antimalarial (Okokon et al., 2005) and antidiabetic activities (Okokon et al., 2006).

Several compounds were isolated from the stem bark by Ngadjui and coworkers: crotonadiol and crotozambefurans A–C together with crotocorylifuran, 7β-acetoxytrachyloban-18-oic acid, trachyloban-7β, 18-diol, lupeol, betulinol, sitosterol and its 3β-glucopyranosyl derivative (Ngadjui et al., 1999; 2002). We isolated five diterpenes from the dichloromethane extract of the leaves: *ent*-trachyloban-3β-ol, *ent*-18-hydroxytrachyloban-3β-ol, *ent*-18-hydroxytrachyloban-3-one, *ent*-trachyloban-3-one and *ent*-18-hydroxyisopimara-7,15-diene-3β-ol, together with transphytol, β-sitosterol, α-amyrin and stigmaterol (Block et al., 2002, 2004). We also reported that the trachylobane diterpene *ent*-trachyloban-3β-ol has a cytotoxic activity on HeLa cells (Block et al., 2002) and is able to induce apoptosis in human promyelocytic leukemia cells *via* caspase-3 activation (Block et al.,

* Corresponding author. Tel.: +32 81 72 42 92; fax: +32 81 72 42 99.
E-mail address: severine.robert@fundp.ac.be (S. Robert).
[1] These authors equally contributed to this work.

2005). We further demonstrated the vasorelaxant activity of *ent*-18-hydroxytrachyloban-3-one, *ent*-18-hydroxytrachyloban-3β-ol and *ent*-18-hydroxyisopimara-7,15-diene-3β-ol (Baccelli et al., 2005, 2007).

Hypertension and thromboembolism are often associated in several cardiovascular and heart diseases such as atherosclerosis (Insull, 2009), heart failure (Gary and Davis, 2008), pre-eclampsia (Inglis et al., 1982) and pulmonary hypertension (Lang and Klepetko, 2008). Consequently, we studied the potential effects of leaf extracts from *Croton zambesicus* on hemostasis. For this purpose, we prepared the dichloromethane and aqueous extracts. The aqueous and dichloromethane fractions from the aqueous extract were also made. The potential effects of these four extracts/fractions were further investigated on red blood cells lysis, on platelet aggregation (primary hemostasis) and on coagulation cascade (secondary hemostasis).

2. Materials and methods

2.1. Plant material, extraction and preparation of fractions

Aerial parts of *Croton zambesicus* were collected in 2005 near Cotonou (Benin) and identified by Pr V. Adjakidjé from the Abomey-Calavi University in Benin. A voucher specimen was deposited at the herbarium of the Belgian National Botanical Garden at Meise (BR S.P. 848.108). Leaves were dried in the dark at room temperature to be further pulverized and kept dry at constant temperature in the dark. For the dichloromethane extract, 70 g powdered air-dried leaves were extracted in Soxhlet with 750 ml CH_2Cl_2 during 6 h (yield 7.7%). For the aqueous extract, 3.5 g powdered air-dried leaves were boiled in distilled water at 100 °C for 20 min. The aqueous extract was further concentrated to dryness (yield 2.93%). Aqueous (yield 1.23%) and dichloromethane (yield 0.90%) fractions were also prepared by liquid–liquid extraction of 100 ml fresh aqueous extract with three times 100 ml CH_2Cl_2.

For the *in vitro* assays, stock solutions at 50 mg/ml were made in dimethylsulfoxyde (DMSO) for the dichloromethane extract and the dichloromethane fraction of the aqueous extract and in a 1:1 mixture ethanol/water for the aqueous extract and the aqueous fraction from the aqueous extract. The working solutions were obtained by dilution with phosphate-buffered saline (PBS, 6.7 mM phosphate, pH 7.4).

2.2. Preparation of human platelet-rich plasma, platelet-poor plasma and 10% (v/v) red blood cells suspension

After informed consent had been obtained, blood from male healthy volunteers, who were free from medication for at least two weeks, was taken by venipuncture and collected into 0.105 M sodium citrate (9:1, v/v) tubes (BD Vacutainer®). Platelet-rich plasma (PRP) was obtained from the supernatant fraction after centrifugation for 10 min at $200 \times g$ at room temperature. The remaining blood was centrifuged at $2000 \times g$ for 10 min at room temperature and the supernatant gave the platelet-poor plasma (PPP). A red blood cells pellet was subsequently obtained by centrifugation at room temperature for 5 min at $3000 \times g$. The pellet was rinsed two times with PBS and a 10% (v/v) red blood cells suspension was obtained by dilution with PBS. The red blood cells suspension was used immediately after preparation. The platelet concentration of PRP was adjusted to 3×10^8 cells ml^{-1} with PPP and PRP was used immediately after preparation. Remaining PPP was frozen at −80 °C immediately after centrifugation and then defrosted at 37 °C for 10 min just before use.

2.3. Hemolysis assays

The hemolysis assays were conducted according to a previously described procedure (Belokoneva et al., 2003). Briefly, 250 μl of 10% (v/v) red blood cells suspension were incubated under agitation at room temperature for 1 h with 5 μl of samples. The mixtures were then centrifuged at room temperature for 5 min at $10,000 \times g$ and the absorbance of the supernatants was measured at 550 nm with a microplate reader Ascent Multiskan EX (Thermo Labsystems). The extracts/fractions were tested in triplicate at a final concentration of 1 mg/ml. Triton X-100 1% (v/v) was used as a positive control (100% red blood cell lysis) and PBS as a negative control (0% red blood cell lysis). The red blood cell lysis percentage (H) was determined as follows: $H = (OD_{550\,nm}$ sample $- OD_{550\,nm}$ PBS$)/(OD_{550\,nm}$ Triton X-100 1% $- OD_{550\,nm}$ PBS$)$. The results were expressed as means $\pm$ SD with $n = 3$.

2.4. Platelet aggregation assays

Aggregation tests were performed according to Born's turbidimetric method (Born and Cross, 1963) with a double-channel aggregometer (Chronolog Corporation, Chicago, IL, USA). PPP was used to adjust the photometric measurement to the minimum optical density. 245 μl of PRP was added in a glass turbidity tube and stirred (1100 rev min^{-1}) at 37 °C. 2.5 μl of samples were then added and the mixture was incubated at 37 °C for 1 min. Platelet aggregation was initiated by addition of 5 μl ADP 200 μM (Helena, final concentration 4 μM) or 10 μl collagen 100 μg/ml (Helena, final concentration 4 μg/ml) or 2.5 μl Protease-Activated Receptor 1 Selective Activating Peptide 500 μM (PAR1-AP, SFLLRN-NH$_2$, Sigma, final concentration 5 μM) or 5 μl U-46619 50 μM (Cayman Chemical, final concentration 1 μM). To evaluate platelet aggregation, the maximum increase in light transmission was determined from the aggregation curve 6 min after addition of the inducer agent. The extracts/fractions solvents were used as control groups. The platelet inhibition aggregation percentage (I) was determined as follows: $I = 1 - $ (platelet aggregation percentage sample/platelet aggregation percentage control group). The results were expressed as means $\pm$ SD with $n = 2$. The extracts/fractions were tested in duplicate at a final concentration of 200 μg/ml.

2.5. Thrombin activity profiles

The thrombin activity measurements were performed using the previously reported Calibrated Automated Thrombogram® (CAT) procedure (Robert et al., 2009). PPP-Reagent 5 pM and Thrombin Calibrator from Thrombinoscope BV were reconstituted with distilled water according to the manufacturer's instructions. For each experiment, a fresh mixture of fluorogenic substrate/calcium chloride buffered solution was prepared as follows: 2275 μl of 20 mM Hepes buffer pH 7.35 containing 60 mg/ml of bovine serum albumin (Sigma) and 260 μl of 1 M calcium chloride were mixed with 65 μl of 100 mM DMSO solution of fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC, Bachem). Dade® Actin® FS Activated PTT Reagent (a mixture of ellagic acid and soybean phospholipids) was obtained from Siemens Healthcare Diagnostics and was diluted 25-fold with distilled water. Argatroban was purchased from Sequoia Research Products. For the tests, 80 μl of PPP, 10 μl of samples (or extracts/fractions solvents alone in the control samples) and 20 μl of PPP-Reagent 5 pM or 20 μl diluted Actin FS® were mixed in a 96-well microtiter plate (Thermo Immulon 2HB) and were incubated for 5 min at 37 °C. The plasma clotting was triggered by the addition of 20 μl of fluorogenic substrate/calcium chloride buffered solution at 37 °C. A calibration curve was also performed for each blood draw using 80 μl of PPP, 10 μl of extracts/fractions solvents, 20 μl of Thrombin Calibrator and 20 μl of substrate/calcium

chloride buffered solution. The reaction of fluorogenic thrombin substrate hydrolysis was monitored on a microplate fluorometer Fluoroskan Ascent FL (Thermo Labsystems) with a 390/460 nm filter set (excitation/emission) using the Thrombinoscope® software version 3.0 (Thrombinoscope BV). The control parameters were expressed as mean ± SEM with $n = 9$. The samples were tested in triplicate at 0.5, 1, 2 and 4 mg/ml for the extracts/fractions and at 0.25, 0.5, 1, 2 and 4 µg/ml for argatroban. The extracts/fractions solvents were used as control groups. The $2\times$ lag time and $2\times T_{max}$ parameters were determined by linear regression whereas the C_{max} EC_{50} and ETP EC_{50} values were calculated using a normalized four-parameter logistic equation with the GraphPad Prism® software (version 5.01). The results were expressed as means ± SD with $n = 3$.

2.6. Enzymatic assays

2.6.1. Thrombin

20 µl of samples (or extracts/fractions solvents in the control samples) and 20 µl human thrombin (Roche, 23.2 nM) in 140 µl 0.01 M Tris–HCl buffer, pH 7.5, 0.01 M Hepes, 0.1 M NaCl, 0.1% (w/v) PEG 6000 were incubated during 10 min in a 96-well assay plate. 20 µl S-2238 (Chromogenix, 2.5 mM) were then added to the mixture. After 5 min of substrate hydrolysis, the reaction was stopped by the addition of 20 µl acetic acid 10% (v/v) and the absorbance at 405 nm was measured in a microplate reader (Multiskan EX, Thermo Electron Corporation). The background absorbance was measured just before adding the enzyme. The extracts/fractions solvents were used as control groups. The samples were assayed in triplicate at 100 µg/ml for the extracts/fractions and at 0.05 µg/ml for argatroban. The enzyme inhibition percentage (I') was determined as follows: $I' = 1 - (OD_{405\,nm}$ sample $- OD_{405\,nm}$ background sample)/($OD_{405\,nm}$ control group $- OD_{405\,nm}$ background control group). The results were expressed as means ± SD with $n = 3$.

2.6.2. FXa

The same protocol as described for thrombin was followed using 20 µl human FXa (Kordia, 58 nM), in 140 µl 0.05 M Tris–HCl buffer, pH 7.5, 0.005 M $CaCl_2$, 0.15 M NaCl, 0.001 M EDTA, 0.05% (v/v) Tween 20 and 20 µl S-2765 (Chromogenix, 1 mM).

2.6.3. TF/FVIIa

The same protocol as described for thrombin was followed using 20 µl human factor VIIa (NovoSeven®, Novo Nordisk, 50 nM) in 140 µl Innovin® (Dade Behring, 130 mg/ml) dissolved with 0.1 M Tris–HCl buffer, pH 7.5, 0.3 M NaCl, 0.01 M $CaCl_2$, 0.1% (w/v) BSA and 20 µl Chromozym t-PA (Roche, 5 mM). In this test, the reaction was stopped after 20 min of substrate hydrolysis.

3. Results

3.1. Hemolytic activity

The potential hemolytic activity of the extracts/fractions was investigated by measuring the lysis of a 10% (v/v) human red blood cells suspension in a spectrophotometric lysis assay. In this experiment, Triton X-100 1% (v/v) was used as a positive control and induced 100 ± 1% of red blood cell lysis whereas the negative control PBS provoked no lysis (0 ± 0%). The extracts/fractions were tested at a concentration of 1 mg/ml and no significant red blood cells lysis (>3 ± 1%) was observed.

3.2. Antiplatelet activity in human platelet-rich plasma

The potential antiplatelet activity of the extracts/fractions was investigated using platelet aggregation studies induced by various agonists: 4 µM ADP, 4 µg/ml collagen, 5 µM PAR1-AP or 1 µM
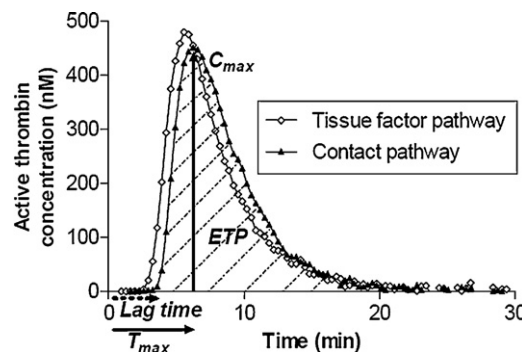

Fig. 1. Control thrombin activity profiles (without extracts/fractions) induced by the contact (white rhombus) or tissue factor (black triangles) coagulation pathways. Data from one experiment shown as means with $n = 3$. The measurements parameters are illustrated for the contact pathway curve.

U46619. The extracts/fractions at 200 µg/ml did not significantly inhibit the platelet aggregation induced by these four platelet agonists (platelet aggregation inhibition < 5% compared to the control groups).

3.3. Anticoagulant activity in human platelet-poor plasma

Thrombin generation assays performed with the CAT method were used to assess the potential anticoagulant activity of the extracts/fractions. The catalytic activity of thrombin was measured with a specific fluorogenic substrate in human plasma during the entire coagulation process. The coagulation cascade was triggered by two different pathways using specific reagents: 5 pM tissue factor (TF) with phospholipids and calcium for the TF pathway and ellagic acid with phospholipids and calcium for the contact pathway.

In absence of the extracts/fractions, the concentration of active thrombin showed a typical peak over time with the two inducers (Fig. 1). This rise of thrombin activity started after a short lag phase (lag time) and reached at T_{max} a maximum concentration C_{max}. The total amount of active thrombin generated (area under the curve) is called endogenous thrombin potential (ETP). The control parameters (i.e. without extracts/fractions) of the thrombin activity profiles induced by the contact and tissue factor coagulation pathways are listed in Table 1.

When the plasma clotting was triggered by the contact or TF pathways, the extracts/fractions dose-dependently reduced the C_{max} and ETP (Fig. 2A–H). The extracts/fractions concentrations which gave 50% of the C_{max} or ETP, called C_{max} EC_{50} and ETP EC_{50} respectively, are presented in Table 2. The more the inhibition parameter is low, the more the extract/fraction is potent to modify the studied parameter. The aqueous fraction and its aqueous phase were the most active samples to decrease C_{max} and ETP whatever the coagulation trigger used.

In addition, for the contact pathway, the extracts/fractions did not change the lag time or the T_{max} (Fig. 2A, C, E and G).

Table 1
Control parameters of thrombin activity profiles induced by the contact and tissue factor coagulation pathways.

Control parameters[a]	Contact pathway	Tissue factor pathway
Lag time (min)	3.40 ± 0.20	2.59 ± 0.16
C_{max} (nM)	401.77 ± 15.44	379.63 ± 22.93
T_{max} (min)	5.77 ± 0.32	5.36 ± 0.12
ETP (nM min)	2302.44 ± 98.56	2116.55 ± 132.73

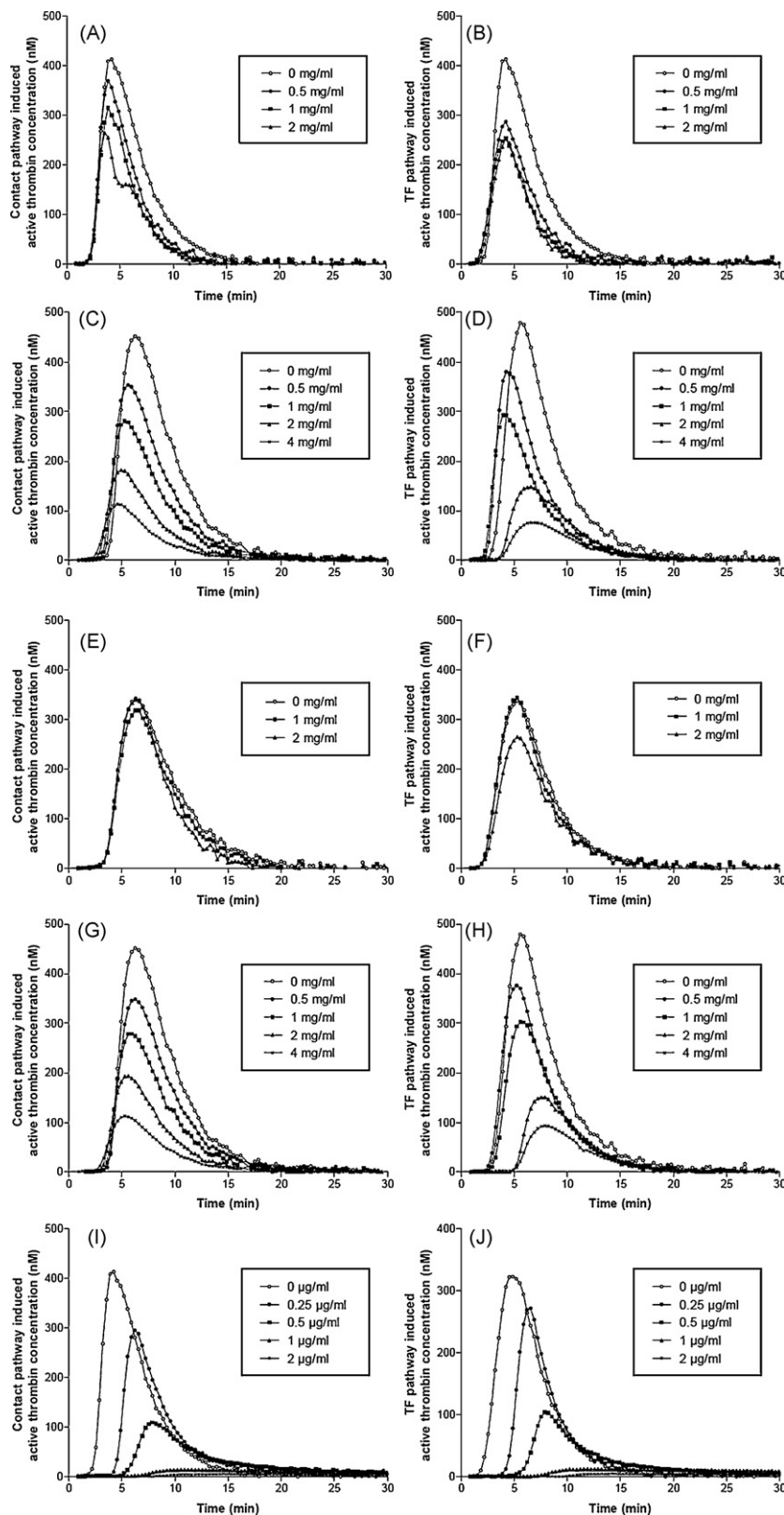[a] Results are expressed as means ± SEM with $n = 9$.

Fig. 2. Effects of the dichloromethane extract (A and B), the aqueous extract (C and D) and the dichloromethane (E and F) and aqueous (G and H) fractions of the aqueous extract from *Croton zambesicus* leaves and argatroban (I and J) on the thrombin catalytic activity during coagulation process induced by the contact pathway (left) or by the tissue factor pathway (right). Data are shown as means with $n = 3$.

Conversely, for the TF pathway, only the aqueous extract and its aqueous fraction dose-dependently slightly increased the lag time and T_{max} (Fig. 2B, D, F, and H). The effective extract/fraction concentrations required for doubling the lag time or the T_{max},

called $2 \times$ lag time and $2 \times T_{max}$ respectively, are listed in Table 2.

The profiles observed in the presence of the fractions/extracts were similar to those obtained in our previous work with the direct

Table 2
Inhibition parameters of extracts/fractions from *Croton zambesicus* leaves and argatroban on thrombin activity profiles.

Products	C_{max} EC$_{50}$ (μg/ml)[a]		ETP EC$_{50}$ (μg/ml)[a]	
	Contact pathway	Tissue factor pathway	Contact pathway	Tissue factor pathway
Dichloromethane extract	2749 ± 153	4825 ± 236	1832 ± 145	2978 ± 200
Aqueous extract	1299 ± 30	1259 ± 30	1273 ± 35	1231 ± 42
Dichloromethane fraction from aqueous extract	7757 ± 3673	7253 ± 108	6859 ± 1605	4055 ± 60
Aqueous fraction from aqueous extract	1304 ± 35	1302 ± 186	1363 ± 65	1399 ± 196
Argatroban	0.35 ± 0.01	0.40 ± 0.01	0.47 ± 0.03	0.42 ± 0.01

Products	2× lag time (μg/ml)[a]		2× T_{max} (μg/ml)[a]	
	Contact pathway	Tissue factor pathway	Contact pathway	Tissue factor pathway
Dichloromethane extract	NE[b]	NE	NE	NE
Aqueous extract	NE	>10,000	NE	>10,000
Dichloromethane fraction from aqueous extract	NE	NE	NE	NE
Aqueous fraction from aqueous extract	NE	5458 ± 140	NE	9441 ± 974
Argatroban	0.39 ± 0.02	0.35 ± 0.01	0.60 ± 0.02	0.64 ± 0.06

[a] Results are expressed as means ± SD with $n = 3$.
[b] No effect.

thrombin inhibitor argatroban (Robert et al., 2009). This anticoagulant agent was consequently used as reference drug in the present study. For the TF or contact pathway, argatroban dose-dependently decreased the C_{max} and ETP and increased the lag time and T_{max} (Fig. 2I and J). At 4 μg/ml argatroban, no thrombin activity was detected. The inhibition parameters for argatroban are also presented in Table 2.

3.4. Inhibitory potency on isolated coagulation factors

We tested the inhibitory potency of the extracts/fractions on isolated blood coagulation factors thrombin (THR), factor Xa (FXa) and tissue factor/factor VIIa complex (TF/FVIIa) in buffer. The extracts/fractions at 100 μg/ml inhibited the amidolytic activity of thrombin, FXa and slightly TF/FVIIa. Argatroban at 0.05 μg/ml only significantly inhibited thrombin (Table 3).

4. Discussion

Natural products from fungi, marine fauna and flora, bacteria and plants are still major sources of innovative therapeutic agents. Current research on natural molecules focuses on plants since they can be sourced more easily and be selected on the basis of their ethno-medicinal use (Cos et al., 2006; Verpoorte et al., 2005). The leaf decoction of *Croton zambesicus* is commonly used in Benin to treat hypertension (Adjanohoun and de Souza, 2002). In previous work, we isolated vasorelaxant diterpenes from leaves of this plant, thus confirming its folk use (Baccelli et al., 2005, 2007). As hypertension and thromboembolism are often linked in a series of pathology, we investigated the potential effects of four extracts/fractions from *Croton zambesicus* leaves on hemostasis: (1) the dichloromethane extract; (2) the aqueous extract; (3) the dichloromethane fraction

from the aqueous extract and (4) the aqueous fraction from the aqueous extract.

Red blood cells are the most numerous types of cells from the blood. They are mainly composed of hemoglobin which enables them to transport oxygen from lungs to all the body tissues throughout the blood circulation. Red blood cells hemolysis is characterised by the breakdown of the red blood cells membrane leading to the release of hemoglobin into the surrounding plasma. This deleterious event can be caused by a large number of conditions and can lead to anemia and hypoxia (Maton et al., 1993). Okokon et al. reported that the administration of the ethanolic leaf extract of *Croton zambesicus* to rats for 21 days produced a dose-dependent reduction of hematocrit, red blood cells count and hemoglobin content (Okokon et al., 2004). Nevertheless, the mechanism by which the ethanolic leaf extract induced anemia (i.e. direct hemolytic activity and/or impaired erythropoiesis) has not been yet elucidated. In the first part of this study, we did not observed any toxic hemolytic activity of the prepared extracts/fractions.

In response to vascular damage, a series of cellular and enzymatic reactions are initiated to create an insoluble fibrin-platelet plug, to arrest blood loss, and eventually to restore vascular integrity. This complex process is called "hemostasis" and is divided in two consecutive stages: the primary hemostasis or platelet aggregation, and the secondary hemostasis or coagulation cascade. The coagulation pathways are depicted in Fig. 3.

In the second part of this work, we excluded a potential antiplatelet activity of the prepared *Croton zambesicus* leaf extracts/fractions by showing that they did not interfere with the platelet aggregation induced by four main potent platelet agonists acting through different signalling pathways.

In the third part of this work, we studied the potential anticoagulant activity of the extracts/fractions using the thrombin activity

Table 3
Inhibition of coagulation factors by extracts/fractions from *Croton zambesicus* leaves and argatroban.

Products	Coagulation factors inhibition[a]		
	THR	FXa	TF/FVIIa
Dichloromethane extract	16 ± 2%	12 ± 6%	13 ± 1%
Aqueous extract	42 ± 1%	31 ± 3%	11 ± 4%
Dichloromethane fraction from aqueous extract	11 ± 3%	6 ± 4%	2 ± 4%
Aqueous fraction from aqueous extract	38 ± 0%	24 ± 4%	9 ± 3%
Argatroban	24 ± 3%	6 ± 3%	0 ± 3%

[a] Results are expressed as means ± SD with $n = 3$; extracts/fractions were tested at 100 μg/ml and argatroban at 0.05 μg/ml.
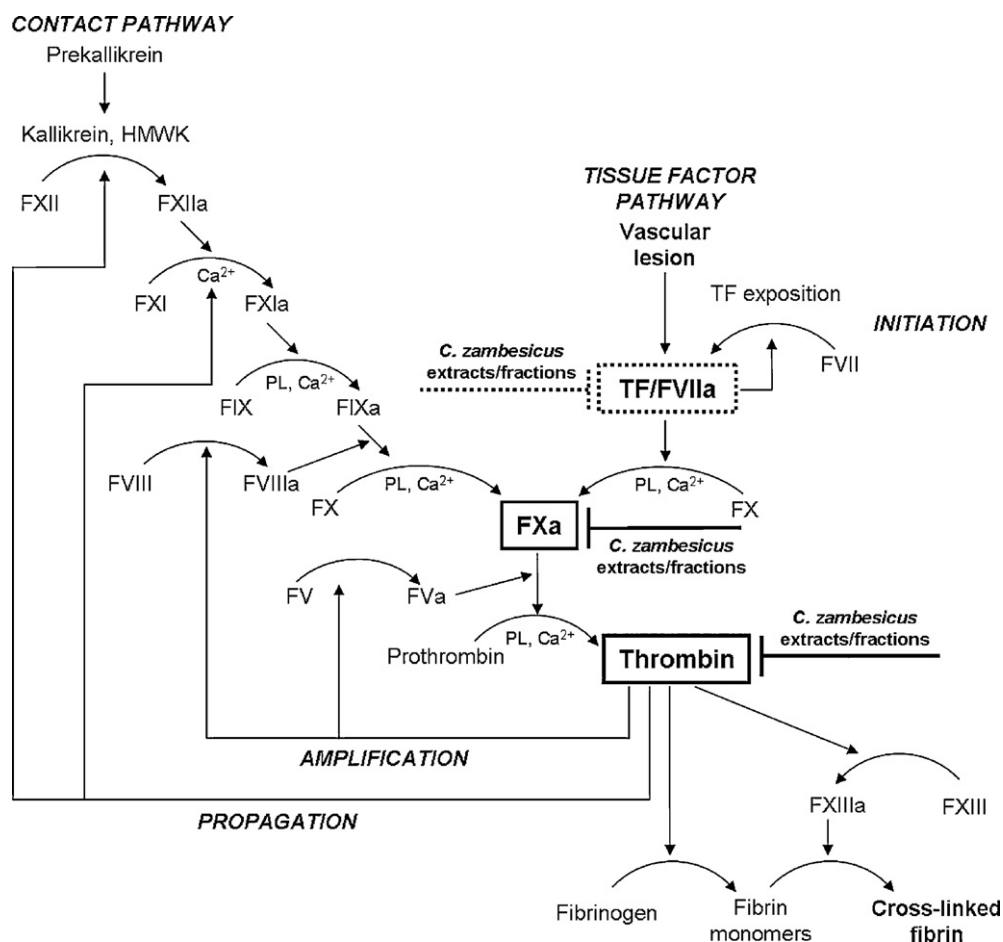
Fig. 3. Coagulation pathways and putative targets for *Croton zambesicus* leaf extracts/fractions. In the initiation phase, the TF/FVIIa complex activates FX. Early free FXa generates small amounts of thrombin, leading to initial fibrin formation. The amplification phase is characterised by the thrombin-induced activation of platelets leading to the exposition of negatively charged platelets phospholipids (PL) and to the release and activation of FV and FVIII. During the propagation phase, thrombin cleaves FXII into FXIIa and FXI into FXIa which in turn activates FIX. The intrinsic tenase, composed of FIXa, FVIIIa, PL and calcium, is formed and activates FX. In the presence of FVa, PL and calcium, FXa assembles into the prothrombinase complex. This latter complex activates prothrombin into thrombin faster than free FXa and induces a burst of thrombin generation. The putative targets of *Croton zambesicus* leaf extracts/fractions are represented in boxes. One or several compound(s) might act on one single or multiple target(s). Thrombin and FXa appear as the major targets of the extracts. An additive but weak effect on TF/FVIIa complex was also demonstrated. Figure adapted from Robert et al. (2009).

profile performed with the CAT method. We previously validated this technique as a pharmacological screening tool by demonstrating that, in the presence of anticoagulant drugs, the thrombin activity profiles were dose-dependently modified according to the specific enzyme inhibitory activity of the drugs (Robert et al., 2009). The advantage of this method is that it may theoretically be applied to any thrombin and/or FXa natural, synthetic or biosynthetic inhibitors whatever their inhibition mode. In the present work, we confirmed that this technique was relevant for the screening of the anticoagulant potential of *Croton zambesicus* leaf extracts. All the experiments were performed with the two specific coagulation triggers for the TF pathway and the contact pathway. The use of these two triggers would allow us to have an insight into the coagulation factors targeted by the extracts/fractions as well as into their specific mechanism of inhibition.

All the control parameters (i.e. without extracts/fractions) were found to be reproducible among the experiments and were in agreement with foregoing studies (Hemker et al., 2003; Robert et al., 2009). In presence of the extracts/fractions, the C_{max} and ETP were dose-dependently reduced when the plasma clotting was triggered by the contact phase or tissue factor pathway, indicating an inhibitory effect on the propagation phase. The most active extracts/fractions were the aqueous extract and its aqueous phase with C_{max} EC_{50} and ETP EC_{50} around 1.3 mg/ml for the two coag-

ulation triggers. As they displayed the same inhibitory potency whatever the coagulation trigger used, it was expected that they act on coagulation factors implicated in the common pathway of the coagulation cascade (i.e. thrombin and/or FXa). They also slightly dose-dependently increased the lag time and T_{max} only for the TF pathway inducer, indicating a minor effect on the initiation phase and therefore, on coagulation factors from the tissue factor pathway (i.e. TF/FVIIa complex). We also compared the thrombin activity profile of the fractions/extracts with those previously obtained with six well-known thrombin and/or FXa inhibitors (Robert et al., 2009) and we chose argatroban as reference drug for the present study due to the similarity of the profiles. This competitive thrombin inhibitor dose-dependently reduced the C_{max} and ETP with EC_{50} around 0.4 µg/ml for the two coagulation pathways inducers. It also dose-dependently increased the lag time and T_{max} with 2× lag time around 0.4 µg/ml and 2× T_{max} around 0.6 µg/ml for the two coagulation pathways inducers. These data were also in agreement with our past study where only 5 pM TF with phospholipids and calcium was used to trigger coagulation (Robert et al., 2009).

The four log difference in anticoagulant activity between the reference drug argatroban and the *Croton zambesicus* aqueous extract should not be considered like a braking issue of this study as the samples display very different and specific composition. Argatroban is a small synthetic molecule of 526.65 g/mol (Kikumoto

et al., 1984) while the *Croton zambesicus* aqueous extract was a crude extract composed of numerous molecules. The anticoagulant activity of the aqueous extract could thus be due to one or more of its constituents present in variable amounts and mainly found in its aqueous phase after liquid–liquid extraction with dichloromethane. Indeed, the aqueous fraction from the aqueous extract was as active as the crude aqueous extract whereas its dichloromethane fraction drastically lost the anticoagulant activity.

In the last part of this work, the effects of the prepared extracts/fractions were assessed on the amidolytic activity of coagulation factors in buffer in order to determine their inhibitory mechanism. The extracts/fractions were found to directly inhibit thrombin and FXa. A weak inhibitory effect on TF/FVIIa complex was also demonstrated. The most active products were the aqueous extract and its aqueous phase. On the contrary, argatroban only significantly inhibited thrombin in accordance with its well-known selective thrombin competitive inhibition mechanism (Kikumoto et al., 1984). From these results, we could also exclude an indirect inhibition of the coagulation factors by the *Croton zambesicus* leaf extracts/fractions, as observed with many natural anticoagulant products (Cipriani et al., 2009; Farias et al., 2001; Grauffel et al., 1989; Guglielmone et al., 2002; Hayakawa et al., 1995; McLellan and Jurd, 1992; Yoon et al., 2002, 2003), as antithrombin III or heparin cofactor II were dispensable to inhibit the amidolytic activity of thrombin and FXa. Furthermore, the thrombin activity profiles of leaf extracts/fractions from *Croton zambesicus* were completely different from those obtained in our previous study with the antithrombin-dependent inhibitors enoxaparin and fondaparinux (Robert et al., 2009). Remarkably, all the data from this *in vitro* assay with isolated coagulation factors were consistent with those obtained with the *ex vivo* thrombin inhibitory profiles in human plasma demonstrating the reliability and the potency of this latter method for screening anticoagulant products.

In our previous work, we studied the vasorelaxant effect of leaf extracts from *Croton zambesicus* which could be related to one of its major therapeutic use in Beninese traditional medicine (Baccelli et al., 2005, 2007). A vasorelaxant activity on 100 mM KCl-induced rat aorta ring contractions was obtained with the dichloromethane extract and the dichoromethane fraction from the aqueous extract at 20 μg/ml whereas no significant vasorelaxant effect was observed with the aqueous extract at the same concentration (data not published). Vasorelaxant diterpenes from the dichloromethane extract of the leaves of *Croton zambesicus* were subsequently isolated and characterised (Baccelli et al., 2005, 2007; Block et al., 2004). It is thus foreseen that the anticoagulant compound(s) of the *Croton zambesicus* leaves are different from the vasorelaxant ones.

From these *in vitro* studies, we can conclude that the prepared leaf extracts/fractions from *Croton zambesicus* present no significant effect on red blood cells integrity or on platelet aggregating capability. However, they display a moderate but significant anticoagulant activity which would be mediated through the direct inhibition of thrombin, FXa and TF/FVIIa complex. Our results also show that the active anticoagulant compound(s) would be mainly present in the aqueous extract and especially in its aqueous fraction. This (of these) anticoagulant compound(s) might act on one single or multiple target(s). Thrombin and FXa appear as the major targets of the extracts. An additive but weak effect on TF/FVIIa complex was also observed (Fig. 3).

This experimental work reported for the first time the anticoagulant effect of leaf extracts from *Croton zambesicus*. From an ethnopharmacological point of view, these findings are of particular interest as the leaves from *Croton zambesicus* are commonly used in infusion by local population. It would be of interest to determine if the chronic oral administration of leaf decoction to animals would influence their primary and secondary hemostasis parameters in such a way that beneficial antithrombotic and/or adverse hemorrhagic effects would be observed. A limited antithrombic effect associated with vasorelaxation may prove to be very interesting for the prevention of cardiovascular diseases, particularly in hypertensive patients which use *Croton zambesicus* leaf decoction. Nevertheless, further *in vivo* studies, and particularly long-term toxicity studies, can be recommended before the use of *Croton zambesicus* as some diterpenes proved to have some cytotoxicity (Block et al., 2004, 2005).

From the pharmacochemist's opinion, phytochemical analysis and isolation of the active compound(s) from the aqueous extract would be carried out with the aim of identifying new anticoagulant prototypes. A complete *in vitro* and *in vivo* pharmacological study would be further performed in order to determine the mechanism(s) of action as well as the potential antithrombotic activity and/or adverse hemorrhagic effects of such isolated molecule(s) from *Croton zambesicus* leaves.

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