Calcium antagonist of n-butanol fraction (BuF) from the stem bark of Terminalia superba Engl. et Diels (Combretaceae) on rabbit duodenum

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Abstract: The Objective of the present study was to investigate the pharmacological effects and the possible mechanism(s) that mediated the effects of the organic fraction from n-butanol-water partition (50:50 v/v) of the stem bark of Terminalia superba and to screen for the presence of phytochemical constituents which can be responsible for these observations. Organic n-butanol fraction (BuF) from the stem bark of Terminalia superba was evaluated for its capacity to induce spasmodic effect on spontaneous contraction of isolated rabbit duodenum as well as for its possible calcium blocking actions. Pieces of rabbit duodenum (3cm) were mounted in an organ bath containing air bubbled Mac Ewen solution with 1g initial tension. The non cumulative concentrations (0.02, 0.04 and 0.08 mg/ml) of BuF (p<0.05). In high potassium (2.43 x10^3 mg/ml) contracted duodenum by ACh (2.20x10^-3 mg/ml), KCl (2.43 x10^6 mg/ml) and BaCl2 (2.30 x10^6 mg/ml) were relaxed by the non cumulative concentrations (0.02, 0.04 and 0.08 mg/ml) of BuF (p<0.05). In high potassium (2.43 x10^6 mg/ml) Ca^{2+}-free Mac Ewen solution, non cumulative concentration of CaCl2 (2.02 x10^{-1} µg/ml) induced duodenum contractions. However, the non cumulative concentrations of BuF (0.02, 0.04 and 0.08 mg/ml) reduced these CaCl2-induced contractions concentration-dependently (p<0.05). These results suggest that BuF antispasmodic effect on rabbit duodenum may be due to blockade of voltage dependent calcium channels. Tannins, flavonoids, quinones, saponins, reduced sugar, sterols and polyterpenes were found out as major active constituents of the tested extract. Some of these phytochemical constituents such as tannins, flavonoids, saponins, sterols and polyterpenes which are known for their antispasmodic effect may explain the relaxant actions of BuF.

Keywords: Antispasmodic, Terminalia superba, rabbit, duodenum.

INTRODUCTION
Terminalia superba Engl. et Diels (Combretaceae) commonly called «fraké» or «limbo» grows from France-Guinea, Congo, Cameroon, Angola to Democratic Republic of Congo [1]. In Côte d’Ivoire, this plant grows in the same place with Triplochiton scleroxylon (Sterculiceae) generally called «samba ». Terminalia superba is widely used for the treatment of various illnesses in folk medicine. The stem barks of the plant are used to heal diseases including abdominal pains, diabetes, diarrhoea, peptic ulcers, female infertility, headache, dysentery, bacterial infections and general fatigue [2-3-4-5-6-7]. Analgesic, antibacterial and endothelium independent vasorelaxant properties were found out [8-9-10]. Although Terminalia superba is used as antigastrointestinal disorders agent in folk medicine, no or few scientific studies were undertaken to explain its actions and usefulness as antispasmodic. The aim of the present study was to investigate the pharmacological effects and the possible calcium antagonist role of the n-butanol organic fraction from the stem bark of Terminalia superba and to screen for the presence of phytochemical constituents which can be responsible for these observations.

MATERIALS AND METHODS

Plant material
The stem barks of Terminalia superba were collected locally from the forest of Ebillassokro village in the East of Côte d’Ivoire in December 2009. Taxonomical identification of the stem barks was established by Professor Aké-Assi Laurent from the National floristic Centre of University of Felix Houphouet Boigny, Cocody- Abidjan, Côte d’Ivoire, voucher n° 2456, Terminalia superba Engl. et Diels in June 4, 1954 ; n° 4207 in March 26, 1957 ; n° 10477, February 26, 1969 and n° 416 in April 03, 1974 of Côte d’Ivoire national herbarium.
Preparation of the extracts

The stem barks were dried under shade and powdered with a machine (mark RETSCH, type SM 100, Germany). The extraction process was implemented according to Ivorian researchers’ method [11]. One hundred grams (100g) of the stem barks powder were macerated during 24 hours in 11 ethanol-water (70:30 v/v) for 3 times until complete exhaustion. The mixtures were filtered (Whatman n°1) and concentrated under reduce pressure using a rotary evaporator (Büchi R110, type MKE 6540/2) at a temperature of 45°C. The concentrated extracts were stored in dessicators at 45°C. Ten grams (10g) of the dried hydroethanol extracts 70% were then partitioned separately in n-butanol (Bu), chloroform (Ch), ethylacetate (Ea) and hexane (Hex)-water (50:50 v/v). Two fractions were obtained from each partition, the organic and the aqueous fractions after decantation, solvent evaporation and storage in dessicators at 45°C respectively. Organic fractions obtained from these partitions were BuF (3.218g), FCh (3.218g), EaF (3.218g), HexF (3.218g) and the aqueous fractions were BuF (6.782g), ChF (6.782g), EaF (6.782g), HexF (6.782g). The concentrations to be tested were prepared extemporaneously by dilution in the Mac Ewen physiological solution (mM): NaCl, 130; KCl, 2.5; CaCl2, 2.4; NaHPO4, 1.18; NaHCO3, 11.9; MgCl2, 0.24; glucose, 2.2 pH=7.4. The n-butanol organic fraction (BuF) was found to be the most interesting fraction because it produced more than 80% (p<0.05) inhibition of contractions induced by rabbit duodenum during the preliminary tests and was retained for pharmacological and phytochemical tests.

Animals

Ten (10) rabbits (Oryctolagus cuniculus) of either sex weighing 2 ±0.2 kg were selected for the experiments. They were bred in the Animal House of the Laboratory of Physiology, Pharmacology and Pharmacopoeia of UFR Sciences of Nature at the University of Nangui Abrogoua (Abidjan, Côte d’Ivoire) according to the principles for the care and use of laboratory animals of the Ethical Committee of the University of Nangui Abrogoua (Abidjan, Côte d’Ivoire). The animals were deprived of food for 24 hours prior to the experiments.

Drugs

Acetylcholine (ACh) was purchased from Prolabo (France). Barium chloride (BaCl2), Calcium chloride (CaCl2), Potassium chloride (KCl) and solutes of Mac Ewen solution were purchased from Sigma (USA).

Duodenum preparations and experimental protocol

All animals were sacrificed by stunning [12-13]. After laparatomy incision, a portion of duodenum (taken within a distance of 5 cm from pylorus) was removed and washed with Mac Ewen solution. Lengths of approximately 3 cm were put in a dish containing the oxygenated physiological solution of Mac Ewen. A selected duodenum strip was placed between two stainless steel hooks vertically in an isolated organ bath thermostated at a temperature of 37°C. The lower hook was fixed to the bottom of the organ bath (150 ml) and the upper one was connected to a C.F. PALMER kymograph transducer [12-13] which transmitted the movements on paper. The initial tension was 1g throughout the experiments. After 60 min, the time necessary for stabilization of the contractile movements, the concentrations to be tested were injected directly into the isolated organ bath containing the oxygenated physiological solution of Mac Ewen. Then the following experiments were performed.

BuF was prepared as 5 mg/mL. A single volume in geometric progression (0.5, 1.0, 1.5, 2.0 and 2.5 ml) of BuF was added in non cumulative manner to the organ bath (150 ml) to obtain concentration-dependent inhibitory effect. The final concentrations of the extract were determined as function of the final volume in the organ bath. The duodenum contractions were induced by final bath concentrations of ACh (2.204 x10^5 mg/ml), BaCl2 (1.18 x10^4 mg/ml) or KCl (2.435 x10^5 mg/ml) and when the plateau was reached, the extract was added non cumulatively to the organ bath, giving final concentrations ranging from 0.02 to 0.08 mg/ml. To show whether the spasmylactic activity of the active fraction was done via a calcium channel blockade, the duodenum was allowed to stabilize in normal Mac Ewen solution, and then was replaced by a Ca^{2+}-free Mac Ewen solution for 30 min. This solution was replaced with final bath concentration of K^{+} rich (2.435 x10^5 mg/ml) and Ca^{2+}-free Mac Ewen solution. Following an incubation period of 30 min and after confirmation of no spontaneous contractions of duodenum, Ca^{2+} was added non cumulatively to the organ bath giving final bath concentrations varying from 7.9x10^5 to 7.9x10^5 mg/ml to obtain control concentration-response curves of Ca^{2+}. Then, the same procedure was repeated with a single final bath concentration of CaCl2 (2.02 x10^5 µg/ml) and when the plateau of contractions was reached, final concentrations of BuF (0.02, 0.04, 0.08 mg/ml) were administered. All the concentrations in the results are final bath concentrations.

Phytochemical screening

The organic fraction of n-Butanol extract from the stem barks of Terminalia superba was screened for the presence of polyphenols, tannins, flavonoids, polyphenols, saponins, alkaloids, sterols and ployterpenes, reduced sugar, proteins, coumarines and quinones. Detection of these constituents was carried out as described by some authors [14]. This experiment was repeated 3 times (n=3).

Data analysis

All values were expressed as mean ± standard error on the mean (m±sem). Statistical analysis and
graphics were carried out using the software GraphPad Prism 5.01 (San Diego, California, USA). The significance of the differences observed between the concentrations was achieved by analysis of variances (ANOVA) of the multiple test of comparison of Tukey-Kramer. The differences between the concentrations were considered statistically significant when p<0.05. The plateau of the contraction caused by each spasmogen (ACh, KCl, BaCl$_2$ and CaCl$_2$) in the absence of the extract was considered as 100% contraction.

**RESULTS**

**Spasmolytic effects of BuF on isolated rabbit duodenum spontaneous contractions**

BuF, at non cumulative concentrations ranging from 0.02 to 0.08 mg/ml, caused a concentration-dependent and significant (p<0.05) decrease of the amplitude and the contractile force of rabbit duodenum contractions. The effects of BuF appeared about 3 min after administration of the substance. The amplitude diminished from 300 ± 40 to 5 ± 0.1 mg. The contractile force decreased from 37 ± 1 to 820 ± 30 mg under baseline. These decreases corresponded to variations from 17.95 ± to 100 % for the amplitude and from 4.50 ±3.1 to 100 % for the contractile force. The EC$_{50}$ values determined graphically (Fig.1) were 0.043 mg/ml (BuF) for the amplitude and 0.055 mg/ml for the contractile force. The spasmolytic effects of BuF were reversible and the spontaneous activity returned to normal after washing the preparations.

**Effect of BuF on duodenum contractions induced by ACh, KCl and BaCl$_2$**

Non cumulative concentrations of BuF (0.02, 0.04, 0.08 mg/ml) reduced significantly (p<0.05) and concentration-dependently after 2.5 min the increase of the amplitude and the force of muscle contraction of rabbit duodenum induced by ACh (2.204 x10$^{-6}$ mg/ml), KCl (2.435 x10$^{-6}$ mg/ml) and BaCl$_2$ (2.303 x10$^{-6}$ mg/ml). The decrease of the amplitude varied from 400 ± 30 mg to 50 ± 1 mg (ACh-induced contractions), 550 ± 40 mg to 110 ± 10 mg (KCl-induced contractions), and 500 ± 20 mg to 100 ± 70 mg (BaCl$_2$-induced contractions). These decreases corresponded to variations of 93.12 ± 2.88% to 12.18 ± 4.11% (ACh-induced contractions), 91.40 ± 3.21% to 19.18 ± 3.75% (KCl-induced contractions), and 83.25 ± 3.49% to 16.16 ± 3.13% (BaCl$_2$-induced contractions). The contractile force diminished from 1390 ± 30 to 170 ± 50 mg (ACh-induced contractions), 1620 ± 80 to 180 ± 10 mg (KCl-induced contractions), 2120 ± 300 to 320 ± 50 mg (BaCl$_2$-induced contractions) corresponding to variations from 85.22 ± 2.41 to -10.76 ± 2.18% (ACh-induced contractions), 95.50 ± 2.89 to 10.43 ± 1.26% (KCl-induced contractions), 75.65 ± 2.53 to 11.58 ± 4.27% (BaCl$_2$-induced contractions) for concentrations ranging from 0.02 to 0.08 mg/ml respectively (Fig.2-A, B and C).
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(FIG 2-A). KCl at 2.435 x 10^{-6} mg/ml /ml (FIG. 2-B) and BaCl2 at 2.303 x 10^{-6} mg/ml /ml (FIG. 2-C) on the rabbit duodenum. The differences between spasmogenic effect of each spasmogen in the absence and in the presence of the extract are shown (*p<0.05, **p<0.001, ***p<0.0001, n=5 (one way ANOVA))

**Effect of BuF on CaCl2-induced duodenum contractions**

The contractile response of the duodenum to CaCl2 solution is shown in Fig. 3-A. In the calcium free high potassium medium, rabbit duodenum contractions were reduced and vanished. The addition of CaCl2 in the range of concentrations from 7.9x10^{-9} to 7.9x10^{-5} elicited an increase of the amplitude (500 ± 10 mg to 1100 ± 500 mg) and the basal tone (10 ± 4 to 2510 ± 120 mg). The EC50 values determined were 1.628 x 10^{-3} µg/ml and 2.406 x 10^{-3} µg/ml respectively (Fig. 3-B).

A single concentration of CaCl2 (2.02 x 10^{3} µg/ml) was used in the calcium free high potassium medium to achieve the dose response effects of BuF. After the stabilization of the effects of CaCl2 (2.02 x 10^{3} µg/ml), BuF was added in the same medium. BuF reduced the CaCl2-induced contractions. Indeed, after 3.5 min, a significant (p<0.05) concentration-dependent decrease of the amplitude and the force of muscle contractions was observed (Fig.3-C) with maximum inhibition at 0.08 mg/ml of BuF. The values varied from 400 ± 50 mg to 80 ± 2 mg (for amplitude), 930 ± 80 to -300 ± 10 mg (for muscle basal tone) corresponding to 92.87 ± 3.96 to 0.2 ± 0.01 % (for amplitude) and 85.03 ± 3.78 to -27.38 ± 3.55 % (for contractile force) for extract concentrations ranging from 0.02 to 0.08 mg/ml (Fig.3-D).

**Fig. 3. Effects of BuF on CaCl2-induced contractions on rabbit duodenum**

**Fig. 3-A**: Spasmogenic effect of CaCl2 on the rabbit duodenum spontaneous contractions in Ca2+-free high K+ Mac Ewen solution-depolarized solution

Control (A0): effects of CaCl2 at 7.9x10^{-9} (A1); 7.9x10^{-7} (A2); 7.9x10^{-6} (A3) and 7.9x10^{-5} (A4) mg/ml in Ca2+-free with high K+ Mac Ewen solution-depolarized rabbit duodenum are indicated by the second arrow.

**Fig. 3-B**: Concentration-response curve for CaCl2 on the rabbit duodenum spontaneous contractions.

**Fig. 3-C**: Spasmogenic effect of non cumulative concentration of CaCl2 (2.02 x 10^{3} µg/ml) on Ca2+-free with high K+ Mac Ewen solution-depolarized rabbit duodenum in the absence (C0) and in the presence of different concentrations of BuF. (C1: 0.02; C2: 0.04 and C3: 0.08 mg/ml)

**Fig. 3-D**: Effect of non cumulative concentrations of BuF (0.02; 0.04 and 0.08 mg/ml) on the rabbit duodenum contractions induced by CaCl2 (2.02 x 103 µg/ml) in Ca2+-free with high K+ Mac Ewen solution. The differences between spasmogenic effect of CaCl2 in the absence and in the presence of the extract are shown (*p<0.05, ***p<0.0001, n=5 (one way ANOVA))
Phytochemical studies

Phytochemical screening of the tested extract showed positive results for Polyphenols, Tannins, Flavonoids, Quinones, Reduced sugar, Sterols and Polyterpenes and negative results for alkaloids, saponins, proteins and coumarines (Table 1).

Table 1. Phytochemical screening of BuF

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Reagents</th>
<th>BuF</th>
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<tr>
<td>Polyphenols</td>
<td>FeCl3 test</td>
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<tr>
<td>Tannins</td>
<td>Stasny test</td>
<td>+</td>
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<tr>
<td>Flavonoids</td>
<td>Cyanidine test</td>
<td>+</td>
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<tr>
<td>Quinones</td>
<td>Borntraeger test</td>
<td>+</td>
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<tr>
<td>Alkaloids</td>
<td>Bouchardat test</td>
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<td>Saponins</td>
<td>Frothing test</td>
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<tr>
<td>Sterols</td>
<td>Liebermann test</td>
<td>+</td>
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<tr>
<td>Polyterpenes</td>
<td>Liemann test</td>
<td>+</td>
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<tr>
<td>Reduced sugar</td>
<td>Tollens test</td>
<td>+</td>
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<tr>
<td>Proteins</td>
<td>Biuret test</td>
<td>-</td>
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<tr>
<td>Coumarines</td>
<td>reaction on the lacto cycle</td>
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-: Negative reaction; +: Positive reaction

BuF induced its spasmylytic effect probably by blocking L-type voltage dependent calcium channels. This suggestion is supported by the existence of L-type VDCCs in the intestinal smooth muscle [26]. The contractions induced by ACh, BaCl2 and KCl are dependent on the entry of Ca2+ into the cells through VDCCs, therefore a substance which can inhibit ACh, BaCl2 and KCl-induced contractions is considered to be a calcium channel blocker [27]. However, it is also possible that BuF could relax the intestinal smooth muscle by interfering with calcium transport across membrane or blocking Inositol Triphosphate mediated Ca2+ from internal stores.

In order to confirm the role of VDCCs blockade as a possible mechanism that mediated the relaxant effect of BuF, depolarization of the duodenum preparation in Ca2+-free high K+ Mac Ewen solution was carried out. The results indicated that BuF significantly inhibited in a concentration-dependent manner the CaCl2-induced contractions of isolated rabbit duodenum. These results corroborated that relaxation observed was primarily mediated by the impedance of extracellular calcium influx via VDCCs as it is done by organic calcium channel antagonists such as verapamil, which inhibits markedly the entry of calcium through VDCCs [16]. However, the inhibitory effect clearly indicates the involvement of VDCCs blockade in the spasmylytic effects of BuF. The results revealed that BuF extract contained tannins, flavonoids, quinones, saponins, reduced sugar, sterols and polyterpenes. Some of these phytochemical constituents such as tannins, flavonoids, saponins, sterols and polyterpenes which are known for their antispasmodic

DISCUSSION

BuF induced a concentration-dependent decrease of amplitude and force of muscle contractions of isolated rabbit duodenum when it was applied in a range of concentrations from 0.02 to 0.08 mg/ml. Furthermore, after washing, this inhibitory activity was totally reversible suggesting that these effects are membrane mediated. This was demonstrated with fruit hydroalcoholic extract of Anethum graveolens (Umbelliferae) on the rat ileum contractions [15]. Moreover, our results are similar to what was obtained with extracts of Piper nigrum (Labiatae) leaf, Buddleja scrodiodes (Scrophulariaceae) and Buddleja perforiata (Scrophulariaceae) aerial parts and Morinda morindoides (Rubiaceae) which were also found to induce spasmylytic effects on the rat ileum, rabbit and guinea pig intestine and on isolated guinea pig ileum respectively [16-17-12].

In order to elucidate the mechanism underlying the inhibitory effects of the plant extract, BuF as calcium antagonist was hypothesized and investigated. It had been reported that the disturbance of L-type Ca2+ channel activity was one of the causes of intestinal motility decrease [18].

This study revealed that BuF can elicit spasmylytic effects on ACh, KCl and BaCl2-induced contractions on isolated rabbit duodenum. The ability of the extract to inhibit ACh, BaCl2 and KCl-induced contractions may indicate that the spasmylytic compounds present in BuF extract are not specific receptor antagonists. It is known that these chemicals have the capacity to mobilize either intracellular or extracellular calcium or both of them and show that BuF could act on those calcium components. KCl is often used as a tool to bypass G-Protein Coupled Receptor (GPCR) stimulation and activates smooth muscle by changing the K+ equilibrium potential and clamping membrane potential at some value above the resting level [19]. It is well known that depolarization induced by high potassium concentration activates L-type voltage dependent calcium channels (VDCCs) [20]. ACh, a neurotransmitter of the parasympathetic nervous system induces contractions by activation of muscarinic receptors which in turn, increase the intracellular calcium through Inositol Triphosphate (IP3) and also by facilitating the inflow of extracellular calcium through the receptor operated calcium channel [21-22-23-24]. In addition, BaCl2 as non selective potassium channel blocker induces depolarization and contraction in smooth muscles possibly by promoting calcium release from intracellular pools [18-25].

The inhibitory effect of BuF, which has been carried out by depolarization in Ca2+-free high K+ Mac Ewen solution, clearly indicates that the extract to inhibit ACh, BaCl2 and KCl-induced contractions may indicate that the spasmylytic compounds present in BuF extract are not specific receptor antagonists. It is known that these chemicals have the capacity to mobilize either intracellular or extracellular calcium or both of them and show that BuF could act on those calcium components. KCl is often used as a tool to bypass G-Protein Coupled Receptor (GPCR) stimulation and activates smooth muscle by changing the K+ equilibrium potential and clamping membrane potential at some value above the resting level [19]. It is well known that depolarization induced by high potassium concentration activates L-type voltage dependent calcium channels (VDCCs) [20]. ACh, a neurotransmitter of the parasympathetic nervous system induces contractions by activation of muscarinic receptors which in turn, increase the intracellular calcium through Inositol Triphosphate (IP3) and also by facilitating the inflow of extracellular calcium through the receptor operated calcium channel [21-22-23-24]. In addition, BaCl2 as non selective potassium channel blocker induces depolarization and contraction in smooth muscles possibly by promoting calcium release from intracellular pools [18-25].

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effect may explain the relaxant actions of BuF [28-29-30].

CONCLUSION
The organic n-butanolic fraction from the stem bark of Terminalia superba (BuF) contains spasmyloytic constituents mediating their effect probably through blockade of Ca\(^{2+}\) influx, which may justify the traditional use of the plant. Therefore, further experiments are required to isolate, purify and characterize the active constituent and to elucidate the exact mechanism of action.

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