

Research Article

Anticholinesterase Properties of *Mareya Micrantha* (Benth) Mull. Arg. (Euphorbiaceae) and its Fractions on the Cholinesterase Model of Isolated Rabbit Duodenum

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Abstract

The study of the anticholinesterase properties of *Mareya micrantha*, a plant used in African pharmacopoeia as a laxative, was carried out on cholinesterases of the rabbit duodenum. At 0.15 mg / ml, the aqueous extract (MAR), its fraction (F5) and its sub-fraction (F52), depending on their state of purity, decrease the catalytic speed of cholinesterases during the hydrolysis of the acetylthiocholine

(ASCh), without significantly influencing the Michaelis constant. The sub-fraction (F52) with the best anti-cholinesterase and myostimulant effects revealed by the bio-guided study is 25% more effective than MAR. A study of the High performance liquid chromatography (HPLC) chromatogram of this sub-fraction F52 shows that this plant is rich in phenolic molecules.

Keywords: Laxative; *Mareya micrantha*; Fraction; Cholinesterases; HPLC

Introduction

Constipation accompanies or announces some pathologies. It manifests in an insufficient frequency of defecation, formation of dehydrated stool, hard and difficult to evacuate. To remedy this, the laxatives available in pharmacies are used. These, like most synthetic drugs, often have side effects. As an alternative, and even more for cultural and economic reasons, the vast majority of the African population uses medicinal plants, an undeniable source of active ingredients. *Mareya micrantha* one of these plants is a shrub, about four meters tall with simple leaves, oblong and shapely. It occurs mainly in central and western Africa (Traoré et al., 2004). In Ivory Coast, is called in the vernacular "Oyia" and "Ochibouo-m'pi" respectively by the Dida and M'Bato. It grows on secondary formations. In traditional medicine, this plant is used in the treatment of gastrointestinal dysfunctions, leprosy, leishmaniasis, malaria, trypanosomiasis, and as an oxytocic agent during difficult deliveries (Guedeguina et al., 1995, Mac Foy et al., 1990; Thes et al., 2011). Previous studies have shown that its aqueous extract, endowed with cholinergic, histaminergic and even laxative activities (Traoré et al., 2004; Meité et al., 2010, Dosso et al., 2013) is also cardio protector (Doumbia et al., 2014); the methanoic extract of this plant has antimicrobial effects; its essential oil is antifungal according to Thes (2014). However, these studies provide little information on the effect of *Mareya micrantha* on cholinesterases, enzymes that play a pivotal role in cholinergic mechanisms. To supplement this need for information, the present study, motivated by the use of this plant as a laxative, will evaluate the anticholinesterase properties of MAR and its fractions based on the cholinesterase model of the isolated rabbit duodenum.

Materials And Methods

Plant Material

The fresh leaves of *Mareya micrantha* were collected in the locality of Akoupé, in June 2007, identified and authenticated by Pr Ake Assi Laurent of the Botany Department of the Félix Houphouët-Boigny University (Côte d'Ivoire). After identification, one specimen was deposited under number 1804 at the herbarium of the National Floristic Center of the Félix Houphouët-Boigny University.

Animal Material

For our pharmacological and enzymatic experiments, we used rabbits of the species *Oryctolagus cuniculus* (Leporidae), from the commune of Abobo (Abidjan, Ivory Coast). They weighed on average 2 kg and were acclimatized under ambient conditions ($28^{\circ}\pm 3^{\circ}\text{C}$) for one week in the animal house of the Biosciences Training and Research Unit (UFR) of the Félix Houphouët-Boigny University of Cocody. The photoperiod is 12/24 hours.

Experimental Device

The experimental device consists of a thermostatically controlled water bath, an insulated organ tank filled with physiological solution of the Mac Ewen type, contained in flasks placed 40 cm from the apparatus. The liquids contained in the vials are placed in polyvinyl catheters and then coils that allow these solutions to warm up to a temperature of 38°C . This liquid supply is controlled by a multi-way selector valve. The insulated organ vessel can be drained through a drain at the bottom of the device.

Methods

Method for the Preparation of Aqueous

Extract of *Mareya micrantha*

The leaves of *Mareya micrantha* were air-dried and then crushed. Eighty grams of the dry powder were mixed with two liters of distilled water. The mixture was shaken for 24 hours at room temperature by means of an agitator (AGIMATIC-N). The macerated mixture was filtered through cotton wool. The filtrate was evaporated using a Buchi rotary evaporator at 70°C. After total evaporation, a powder is obtained, part of which is used as an aqueous extract (MAR) and the other part is fractionated.

Method of Partitioning Aqueous Extract Fractionation

The aqueous extract of *Mareya micrantha* (1.5 g) is dissolved in one litre of 70% ethanol (Ethanol/water; 70/30; v/v). The mixture is stirred for 6 hours and left to stand in a separating flask for 24 hours. A hydroalcoholic supernatant (F1) and a precipitate (P) were obtained, collected separately and then evaporated using a Buchi rotary evaporator at 60°C. The resulting pellets are dried in an oven at 50°C. A portion of F1 taken up with a cyclohexane-water mixture (5:5; v/v) is subjected to the same process. A cyclohexane fraction (F2) and an aqueous fraction (F3) are obtained. The extract obtained from the aqueous phase (F3) is taken up in an ethyl acetate-water mixture (5:5; v/v). The solution undergoes the same treatment as the other mixtures. An ethyl acetate fraction (F4) and an aqueous fraction (F5) are obtained. F5 with 1371.42 mg strength revealed by the bio-guided study, presents the best myostimulant effect. It will therefore be retained for solid-liquid fractionation.

Method for Solid-Liquid Fractionation on Sephadex G25 gel from F5

800 mg of fraction F5 dissolved in 10 mL of distilled water is passed over a column (50 cm x 1.5 cm) of Sephadex G25 gel with distilled water as the mobile phase. The rate of elution was adjusted to 2 mL per minute. Volumes of 10 mL were collected and then grouped based on their ccm profile into three sub-fractions F51, F52 and F53. These fractions were evaporated at reduced pressure using a BÜCHI 461 Water Bath rotary evaporator. The powders obtained were used for physiological tests and the F52 with the best positive inotropic effect is analysed using the RP-HPLC (C18) preparative apparatus.

Method for the Preparation of Enzyme Extract

The method of preparation of the enzyme extract is based on that of Khoa and Ochillo (1987) (Khoa et al., 1987), taken up by Bahi (1998). After a 24-hour fast, the rabbit is stunned by a club blow on the neck and bled rapidly through the carotid artery. Following a midline laparotomy, four grams of intestine (duodenum) is immediately removed and immersed in 0.5 M mono and di-sodium phosphate buffer, pH 7.8, and then crushed with a T 25 micro-mill at 2500 rpm for 2 minutes. The grind obtained is then centrifuged at 2500 rpm for half an hour in an Alresa centrifuge. The supernatant obtained constitutes the enzyme preparation.

Method of Preparing Ellman's Reagent for Enzyme Assays

The study of total cholinesterase activity is performed with Ellman's substrate reagent, acetylthiocholine (ASCh) at 0.014 M concentration prepared with 0.4 g acetylthiocholine iodide dissolved in 100 mL phosphate buffer. Ellman's reagent is prepared extemporaneously with the mixture of 100 µL of 0.014 M acetylthiocholine iodide, 100 µL of 0.01M DTNB (Dithiobisnitrobenzoate) (obtained by dissolving 0.4 g of DTNB in 100 mL of 0.5 M

phosphate buffer, pH 7.8) and 800 μ L of 0.5 M phosphate buffer, pH 7.8.

Method for Studying the Activity of Rabbit Duodenum Cholinesterases in the Presence and Absence of the Effector

For the study of cholinesterase activity, 100 μ L of Ellman's reagent with increasing concentrations of acetylthiocholine (10^{-5} M to 10^{-2} M) is added to the reaction medium. This medium consists of 1 mL of phosphate buffer (0.5 M, pH 7.8) and 50 μ L of enzyme solution pre-incubated at 38°C for 5 min. During 10 min incubation, cholinesterases in the presence or absence of 30 μ L of effector at 0.15 mg/mL, hydrolyze acetylthiocholine (of varying concentrations) from the Ellman reagent. The hydrolysis products are acetate and thiocholine, which reacts with DTNB to give a yellow coloration, the intensity of which is measured by spectrophotometer at 412 nm against a control containing no enzyme solution. The change in optical density (OD) relative to the control, measured and plotted against the incubation time (dA/min), represents the initial rate. The LINERWEAVER-BURK representation ($1/V$ as a function of $1/S$) is constructed from these values and used to determine V_{max} and KM .

Method of Removal and Placement of the Isolated Organ

The rabbit who fasted for 24 hours is knocked out. Following a midline laparotomy, 3 cm long segments of duodenum are immediately removed and kept alive in a glucose- and oxygenated Mac Ewen solution. One of the fragments is mounted on the recording apparatus in a well containing 250 ml of Mac Ewen in (mM) NaCl 130; KCl 2.5; $CaCl_2$ 2.40; NaH_2PO_3 1.18; $NaHCO_3$ 11.90; $MgCl_2$ 0.24; glucose 2.2 of pH 7.4 continuously oxygenated and temperature 38°C.

With the aid of a wire passed through the wall of the duodenum fragment, a knot is made at one end of the duodenum fragment enabling it to be hooked into the interior of the insulated organ vessel. The other end is connected by another wire to the stylus, the nib of which is in contact with a paper coated with black smoke, wound on a cylinder subjected to a rotation of constant speed.

Experimental Protocol

Using a graduated syringe, a volume of the dilutions made from the test substances are introduced into the single-organ vessel, a well containing 250 ml of Mac Ewen. To move from one test to the next, the preparation is washed thoroughly with Mac Ewen (750 ml) to avoid the cumulative effect of the test substances.

Method for the Analysis of the Phenolic Constituents of the F52 Sub-Fraction

For the analysis of the phenolic constituents of the F52 fraction, we used the varian RP- HPLC (C-18) apparatus, equipped with a PDA (photo diode array) detector. The injection volume is 20 μ l, the analysis temperature is 25°C and the pump flow rate is 1mL /mn. The mobile phases used during this analysis are: water at 0.1% TFA for eluent A and acetonitrile for eluent B. The different gradients used for the analysis are: mobile phase [T=0: 95% (A), 5% (B)]; [T=10: 85% (A), 15% (B)]; [T=45 70% (A), 30% (B)]; [T=50: 0% (A), 100% (B)]; [T=55: 0% (A), 100% (B)].

Statistical Analysis

Statistical data expressed as mean \pm standard error ($M \pm SEM$) were obtained from (n=4) separate experiments. The calculated means were compared from the Student (t) test. When $p \leq 0.05$, the difference is said to be significant. The curves and

statistical analysis were performed using Graph Pad Prism 5.01, San Diego, CA, USA.

Results

Influence of MAR on Total Cholinesterase Activity of Rabbit Duodenum

Figure 1. shows the effect of MAR at 0.15 mg/mL on the catalytic activity of total cholinesterases in the

rabbit duodenum. In the presence of MAR, the maximum rate and affinity constancy values are $83.3 \mu\text{M}/\text{min}$ ($P < 0.001$) and $8.69 \pm 1.7 \mu\text{M}$, respectively, while in the absence of an effector, V_{max} and K_{M} are $125 \pm 1.3 \mu\text{M}/\text{min}$ and $8.69 \pm 0.9 \mu\text{M}$, respectively. In the presence of MAR, K_{M} does not change while V_{max} decreases significantly ($P < 0.001$), indicating that MAR is a cholinesterase inhibitor.

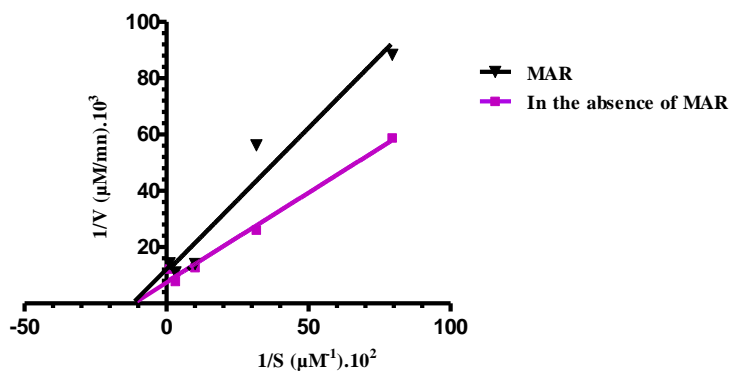


Figure 1: Determination of V_{max} and K_{M} of cholinesterases in the presence of aqueous extract of *Mareya micrantha*

MAR decreases V_{max} without modifying the K_{M} of cholinesterases.

*The values express a variation of K_{M} and V_{max} of cholinesterases in the presence and absence of MAR and its F5 fraction (SEM \pm mean, *** $P < 0.001$; $n=4$).*

Influence of F5 on Total Cholinesterase Activity of Rabbit Duodenum

Table 1. represents the effect of F5 at 0.15 mg/mL on the catalytic activity of total rabbit duodenal cholinesterases. The maximum rate (V_{max}) and affinity constancy (K_{M}) values are $66.6 \mu\text{M}/\text{min}$ and $8.69 \pm 0.9 \mu\text{M}$ in the presence of F5 versus $125 \pm 1.3 \mu\text{M}/\text{min}$ and $8.69 \pm 0.9 \mu\text{M}$ in the absence of an effector, respectively. The V_{max} value decreases significantly ($P < 0.001$) in the presence of F5 while the K_{M} values do not change. The hydrolysis activity of ASCh by cholinesterases decreases. Thus, F5 is an inhibitor.

Table 1 : Kinetic parameters of rabbit duodenum cholinesterase in the presence and absence of F5

Kinetic parameters of cholinesterases in the presence and absence of the effector	K_M (μM)	V_{max} ($10^1 \mu M/mn$)
Cholinesterase	$8,69 \pm 1,5$	$12,50 \pm 1,3$
Cholinesterase+ MAR	$8,69 \pm 1,7$	$8,33 \pm 1,2^{***}$
Cholinesterase+ F5	$8,69 \pm 0,9$	$6,66 \pm 0,97^{***}$

Like MAR, F5 decreases V_{max} without modifying the K_M of cholinesterases.

*The values express a variation of K_M and V_{max} of cholinesterases in the presence and absence of MAR and its F5 fraction (SEM mean, $^{***}P < 0.001$; $n=4$).*

Effect of F5 Fractions on the Contractile Activity of the Rabbit Duodenum

Chromatographic fractionation of F5 on sephadex G25 gel yielded fractions F51, F52 and F53. Figure 2 shows the effect of these fractions on the contractile activity of the rabbit duodenum. F52 and F53 at $100 \mu g.mL^{-1}$ show myostimulant effects, whereas F51 at the same concentration has myorelaxing properties. F52 and F53 increase contraction by 2428.57 ± 5 mg strength ($P < 0.001$) and 534.74 ± 5 mg strength ($P < 0.05$), respectively.

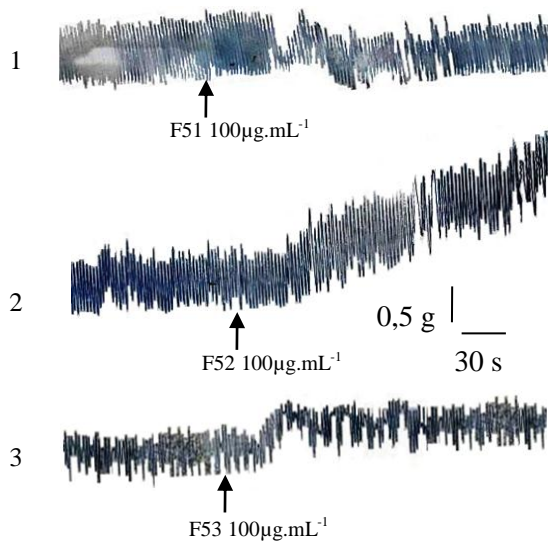


Figure 2: Effect of F5 fractions on the contractile activity of the rabbit duodenum:
 1-Effect of F51 $100 \mu g.mL^{-1}$ 2-Effect of F52 $100 \mu g.mL^{-1}$ 3-Effect of F53 $100 \mu g.mL^{-1}$;
F5 has a dose-increasing myostimulant effect

Figure 3 shows the percentage change in rhythmic contraction of the rabbit duodenum as a function of the F51, F52 and F53 fractions tested at $100 \mu\text{g.mL}^{-1}$. F52 induces the greatest myostimulant effect ($P < 0.001$).

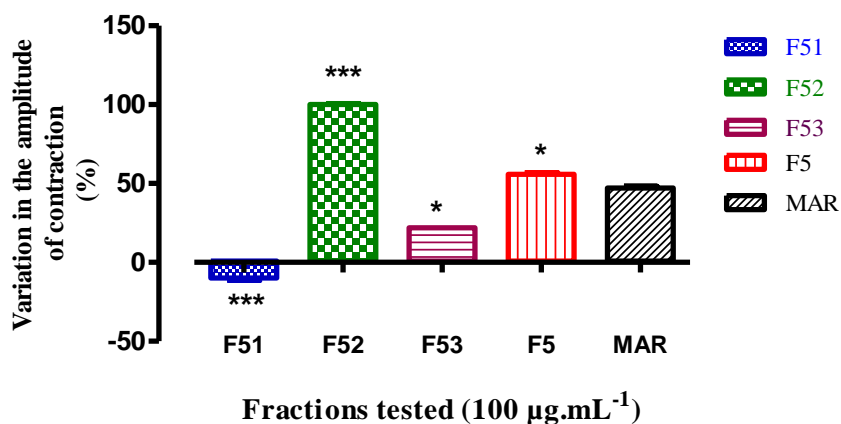


Figure 3: Variation of the amplitude of contraction induced by MAR and its fractions (F5, F51, F52 and F53). F52 induces the most important myostimulant effect.

These values express a variation in contraction as a function of the fraction of F5 tested (SEM mean, $*P < 0.05$; $***P < 0.001$, $n = 4$).

Influence of F52 on total Cholinesterase Activity of Rabbit Duodenum

In the presence of F52 at 0.15 mg/mL the affinity constant and maximum rate V_{max} of rabbit duodenal cholinesterases are $8.69 \pm 0.9 \mu\text{M}$ and $62.5 \mu\text{M/min}$ ($P < 0.001$) respectively, compared to $8.69 \pm 0.9 \mu\text{M}$ and $125 \pm 1.3 \mu\text{M/min}$ in the absence of an effector. V_{max} decreases in the presence of F52 ($P < 0.001$) while K_{M} does not vary in the presence of the effector. The hydrolysis activity of acetylthiocholine (ASCh) by cholinesterases decreases, indicating that F52 is an inhibitor. It is 25% more effective than MAR.

High Performance Liquid Chromatography (HPLC) Chromatograms of F52

The RP-HPLC (C-18) chromatogram of sub-fraction F52 indicates that the aqueous extract of *Mareya micrantha*, is rich in phenolic molecules in view of the peaks recorded. Some of these molecules have been identified, they are coumarins (isoscopoletin), phenolic acids (hydroxy-benzoic acid, ferulic acid, o-coumaric acid) and flavonoids (epicatechin gallate, oriental) (Figure 4).

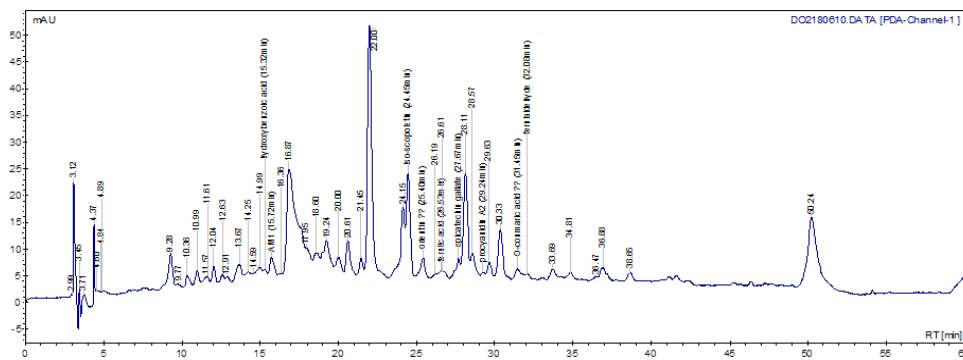


Figure 4: HPLC chromatogram (C-18) of the fraction F52 of *Mareya micrantha*. The F52 fraction contains phenolic compounds, some of which have been detected. These are: isoscopoletin, hydroxy-benzoic acid, ferulic acid, o-coumaric acid, epicatechin gallate, oriental.

Discussion

The study of the anti-cholinesterase properties of *Mareya micrantha* was carried out on rabbit duodenum cholinesterases. It showed that in the presence of the aqueous extract of *Mareya micrantha*, the maximum rate (V_{max}) of hydrolysis of acetylthiocholine (ASCh) from cholinesterases decreased significantly, without changing the Michaelis constant (K_M). These data reveal that the aqueous extract of *Mareya micrantha* has anticholinesterase properties. It is believed to be a non-competitive inhibitor of cholinesterases. This effect of *Mareya micrantha* could be explained by the binding of the anticholinesterase effector molecules of this natural essence at sites different from those of the substrate (ASCh) at the enzyme level. The resulting ternary complex of enzyme, effector and substrate would therefore prevent conformational adjustment, a prerequisite for catalysis at the active site of the enzyme (Wilson et al., 1950; Froede et al., 1986; Diopoh, 2016). Similar mechanisms of competitive inhibition of cholinesterases have been demonstrated by Gnahoue

(2009), using the aqueous extract of *Trema guineensis*.

To identify the phenolic molecules responsible for the anticholinesterase and even laxative properties of this natural essence, its aqueous extract was partitioned into different solvents. The aqueous fraction F5, more spasmogenic than MAR (Dosso et al., 2017), was retained and subjected to a new solid-liquid fractionation on sephadex G25 gel. F52 is the sub-fraction with the best positive inotropic effect. Like MAR, the F5 fraction and this F52 sub-fraction, without affecting the Michaelis constant, reduce the catalytic rate of cholinesterases in a purity-dependent manner. It is deduced that they have become progressively enriched in anti-cholinesterase substances and could act according to the same mechanism as MAR. Inhibit cholinesterases, increase intestinal contraction (Traoré et al., 2004, Dosso et al., 2013). Facilitate intestinal fluidity with electrolytes (Méité et al., 2010), probably via the activation of calcium-dependent channels and *CFTR*, a glycoprotein located at the apical membrane of the intestine that governs ionic movements (chlorine,

sodium, water) (Hirota et McKay, 2006; Frizzell et Hanrahan, 2012). The RP-HPLC (C-18) chromatogram of the F52 fraction confirms the presence of phenolic molecules in this fraction. They are epigallocatechin galate, oriental, scopoletin, ferulic acid which could be endowed with anticholinesterase or even laxative effect. However, further purification studies could make it possible to clearly identify the molecules responsible for the plant's so-called effects. Plants containing phenolic molecules with anticholinesterase effects have been studied: *Cynanchum atratum* and *Origanum majorana* (Somboro et al., 2013).

It can be concluded from this study that the aqueous extract of *Mareya micrantha*, as well as its fractions (F5 and F52) show anticholinesterase activities, they are non-competitive inhibitors; indeed, they decrease the catalytic speed of cholinesterases and have no significant influence on the consistency of Michaelis. These anticholinesterase properties of *Mareya micrantha* and its fractions, probably due to the phenolic content of this natural essence, could partly justify the use of this plant as a laxative in traditional medicine.

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