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Cholinergic and anticholinesterase activities of total protein extract of *Morinda morindoïdes* on isolated rabbit duodenum

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ABSTRACT

Traditional herbal medicines such as *Morinda morindoïdes* are used for treatment of intestinal disorders including constipation in Ivory Coast. The aim of present study was to investigate the effect of total protein of *Morinda morindoïdes* extract (PT-Mm) on rabbit duodenum contractility and the involved possible mechanism(s). PT-Mm was extracted according to the saturation method of Dawson with ammonium sulfate. The cholinergic effect of the extract was determined by the *in vitro* organ bath method. The acetylcholinesterase (AChE) extracted from rabbit duodenum and it activity was determined by Ellman's assay using acetylthiocholine (ASCh) as substrate. PT-Mm concentrations (40, 80, 120 and 200 µg/mL) showed dose-dependent effect a both tonicity and amplitude of the duodenum spontaneous contractions. The effective concentration which induces 50% effect of PT-Mm (EC_{50}) was obtained with 68.57 ± 0.89 µg/mL. The antagonist tests carried out showed a considerable reduction (90%) in the amplitudes of duodenal contractions in the presence of atropine, but with nifedipine, the contractions were completely inhibited. PT-Mm also exerted non-competitive inhibition on AChE (Vmax = 5687 mM/min and K_M = 578 µM). These results suggest that PT-Mm could stimulate duodenum smooth muscle contraction because it contains anti-AChE and cholinomimetic substances which, through muscarinic receptors, increase Ca^{2+} mobilization from extracellular. Therefore, PT-Mm could be used as a laxative, due to its stimulating effects on duodenal contractility.

Keywords: *Morinda morindoides*, acetylcholinesterase, duodenum, contraction.

INTRODUCTION

Many people nowadays turn to the use of natural products for treatment of gastro-intestinal disorders including diarrhoea, indigestion and constipation (Ragone et al., 2007). Constipation is a highly prevalent often chronic gastro-intestinal disorder that affects adults (Bosshard et al., 2004; Muller-Lissner, 2009). Natural products have served as a source of medicine for centuries and about half of the pharmaceuticals in use today are derived from natural products (Ginsburg and Deharo, 2011). Dependance on plants as the source of medicines is prevalent in developing

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countries where traditional medicine plays a major role in health care (Azam et al., 2011).

Morinda morindoïdes (baker) Miln-Redh (Rubiaceae) is well known in the traditional medicine practice of tropical countries. In the Democratic Republic of Congo, *M. morindoïdes* has long been used in villages and towns in the treatment of some parasitic diseases, and the leaf extracts of the plant have been shown to possess antiprotozoal activity particularly against *Entamoeba histolytica* and rheumatic pains (Cimanga et al., 2003; Cimanga et al., 2006). In Ivory Coast, *Morinda morindoïdes* is used as an antifungal agent and to treat diarrhoea (Meite et al., 2009). Recently Zirihi et al. (2005) showed the activity of the ethanol extract of *M. morindoïdes* against chloroquine-resistant FcB1/Colombia strain of *Plasmodium falciparum*. Also, ten flavonoids and eight iridoid glycosides have been isolated from the butanol and ethyl acetate fractions by Cimanga et al. (1999). Some interesting biological activities related to some of its traditional uses, including antioxidative (Cimanga et al., 1999), cardioinhibitory (N'Guessan et al., 2002), anticomplementary (Cimanga et al., 2003), antiamoebic (Cimanga et al., 2006), immunologic (Mankele et al., 2006), antimalarial (Cimanga et al., 2008) and spasmolytic (Cimanga et al., 2010) activities were previously reported. The present work was planned to examine *in vitro* the laxative activity of total protein extract of *Morinda morindoides* and its mechanism on duodenal smooth muscle contractility.

MATERIALS AND METHODS

Plant materiel

The leaves of *Morinda morindoïdes* (Rubiaceae) were collected from Daloa (central west region of Ivory Coast) in June 2009. The plant was identified and authenticated by Pr Ake Assi of the Department of Botany, University of Cocody. A voucher specimen (no 17710) of the plant was deposited in the herbarium of the National Floristic Center of the University of Cocody-Abidjan.

Animals

Rabbits of both sexes, 12-16 weeks old, weighing 1.5-2 kg and bred at the Department of Biosciences, (University of Cocody-Abidjan), were used for the experiments. All animals were kept at constant humidity (60%) and temperature (25 °C) in a 12- hour light / dark cycle. They had free access to food and water.

The animals were cared for and treated according to the principles for using of laboratory animals, and approval for the studies was given by the ethical committee of the University of Cocody- Abidjan. The equipment, handling and sacrificing of the animals were in accordance with the European Council legislation 87/609/EEC for the protection of experimental animals (Mitjans et al., 2008). Before the experiment rabbits were deprived from food for 24 h but had free access to water.

Drugs and chemicals

The drugs used were: atropine, nifedipine, DTNB, acetylthiocholine and ammonium sulfate. All chemicals were purchased from Sigma Chemicals Co. (St Louis, MQ, USA), Aldrich Chemical Co. (Steineheim, Germany), and Merck (Darmstadt, Germany).

Preparation of PT-Mm

M. morindoïdes leaves were cleaned of extraneous matter, air-dried at room temperature for 7 days and ground into a fine powder. The powder was mixed with distilled water (80 g of powder in 2 L of distilled water) for 24 h with constant stirring .The

extract was filtered twice through cotton wool, then through Whatman filter paper (No.1).

The filtrate is saturated with ammonium sulfate to 90% saturation according to the method of Dawson et al. (1986) for the precipitation of protein. After homogenization, the solution was kept for 24 hours and subsequently centrifuged 4000 trs/min. A fraction that was obtained showed two phases: an upper phase (supernatant) and a lower phase (the sediment) which was our protein extract. The sediment collected was dialyzed against distilled water (Wilson and Walker, 1994), with a synthetic membrane of dialysis. The presence of protein in the dialyzed pellet was revealed by the Lowry test (Lowry et al., 1951). Protein solution was freeze-dried for better storage stability (Osterlund and Janson, 1997) and the resulting powder was our total proteins extract of *Morinda morindoides* leaves (PT-Mm).

Duodenum tissue preparation

On the day of experiment, rabbits were sacrificed by a sharp blow on the neck. After a median laparotomy, duodenal muscle strips (2 cm) were dissected and mounted in an organ bath containing Tyrode solution (100 mL) between two stainless steel hooks vertically. The lower hook was fixed at the bottom of the organ bath and the upper one was connected to an isotonic transducer (Harvard transducer, UK) connected to a recorder (Harvard Universal Oscillograph, UK). The Tyrode solution composition (pH 7.4 and 37 °C) was (in mM): NaCl (130.5); KCl (5.63); $CaCl_2$ (2.16); $MgCl_2$ (0.24); NaH_2PO4 (1.18); $NaHCO_3$ (11.90) and glucose (11.10) which was continuously bubbled with air (Madeira et al., 2002). The initial tension was 1 g throughout the experiment and equilibrium period was 30 min. After equilibrium period spontaneous contractions were recorded for 5 min in the absence (control) or presence of increasing doses (40 µg/mL to 200 µg/mL) of PT-Mm. Each test was repeated three times and duodenum strip was washed 2-3 times with the Tyrode solution in order to avoid the cumulative effects of products. Antagonist tests with atropine and nifedipine were made at the concentration for which these substances have no effect on the contractile activity of rabbit duodenum. Thus, different doses of antagonist were assessed on contractile activity of duodenal smooth muscle in the presence of PT-Mm.

***In vitro* analysis of AChE activity**

Enzyme extraction was performed according to the method of Khoa and Ochillo (1987). A length of duodenum weighing 1g was added to 50 mL phosphate buffer and crushed with a mortar (Ultra Turax T 25). The homogenate was centrifugated and the supernatant was used for the assays. The AChE activity was determined *in vitro* by Ellman et al. (1961) method. The assay contained a mixture of 100 µL of 5,5'- dithio-bis-(2-nitro) benzoic acid (DTNB) (0.01 M) and 25 µL of acetylthiocholine (ASCh) of varying concentrations in 50 mM potassium phosphate buffer, pH 7.8, followed by the addition of 75 µL AChE in 50 mM potassium phosphate buffer, pH 7.8. The enzymatic reaction was initiated at 25 °C and the absorbance change was monitored at 412 nm with a spectrophotometer (Amresa, Barcelona, Spain).

Enzyme kinetic analysis

To study the effect of PT-Mm extract on AChE activity, the kinetic analysis of the duodenum AChE solution in the presence of the extract was performed. The mixture of enzyme and PT-Mm (1.5 mg/mL) was preincubated at 37 °C for 5 min, and then the substrate in varying concentrations was added and immediately stirred for 10 s. The change

of absorbance at 412 nm was monitored and the initial velocity (dA/min) of the reaction was calculated from the absorbance change. The kinetics of AChE in the presence of PT-Mm was determined by the Lineweaver and Burk (1934) (LB) plot. The LB plot represents the reciprocal velocities substrate concentrations of the control (without inhibitor) and the series of inhibitor concentrations (Trevor, 1981).

Statistical analysis

Data were analyzed by one-way ANOVA followed by Dennet's t-test using instat (Graph Pad software, USA). A p value of < 0.05 was considered statistically significant.

RESULTS

Dose-response effect of PT-Mm on rabbit duodenum

Figure 1 shows the recordings of the mechanical activity of the rabbit duodenum in the presence of PT-Mm. In this study, the different doses of the extract (40, 80, 160 and 200 µg/mL) showed dose dependent increase on duodenal contractions. The recording obtained at 40 µg/mL showed a no significant ($p > 0.05$) increase in the amplitude of the spontaneous contractions (Figure 1A). At 80 µg/mL of PT-Mm, the increase in the amplitude of duodenal contractions was significant ($p < 0.05$) (Figure 1B), but it was very significant ($p < 0.01$) at the dose of 120 µg/mL of the extract (Figure 1C); while at 200 µg/mL of PT-Mm the amplitude of contractions reached the maximum (100 %) ($P < 0.01$) (Figure 1D).

The results presented on Figure 2 showed the dose-response curve of duodenum contractions induced by increasing doses of PT-Mm. The EC_{50} was determined from the curve of the amplitude to be 68.57 ± 4.89 µg/ml.

Characterization of the active compounds of PT-Mm

Antagonism effect of atropine and PT-Mm on duodenal contractility.

Figure 3 shows the interaction between PT-Mm and increasing concentrations of atropine (muscarinic receptor blocker). The gradual increase in the amplitude of contractions recorded with PT-Mm extract was inhibited significantly (81.25% inhibition) by atropine (4.10^{-6} mg/mL).

Antagonism effect of nifedipine and PT-Mm on duodenal contractility

Figure 4 expresses the interaction between PT-Mm and the varying concentrations of nifedipine (calcium channels blocker). The contractile effect of PT-Mm on spontaneous contractions of duodenal smooth muscle was completely inhibited by nifedipine at 1 µg/mL.

Effect of PT-Mm on hydrolytic action of AChE

The kinetic analysis of AChE inhibition by PT-Mm (40 µg/mL) was shown in Figure 5. PT-Mm inhibited AChE in non-competitive manner. The chart of Lineweaver and Burk (1934) in which the lines obtained cross the Y-axis and the X-coordinates in two distinct points which correspond respectively to $1/Vmax$ and $-1/K_M$ led to the determination of Vmax and K_M. The K_M and Vmax values for AChE in the absence of PT-Mm were 578 µM and 6664 mM/min, respectively. In the presence of PT-Mm the corresponding values were: K_M = 578 µM and Vmax = 5687 mM/min.

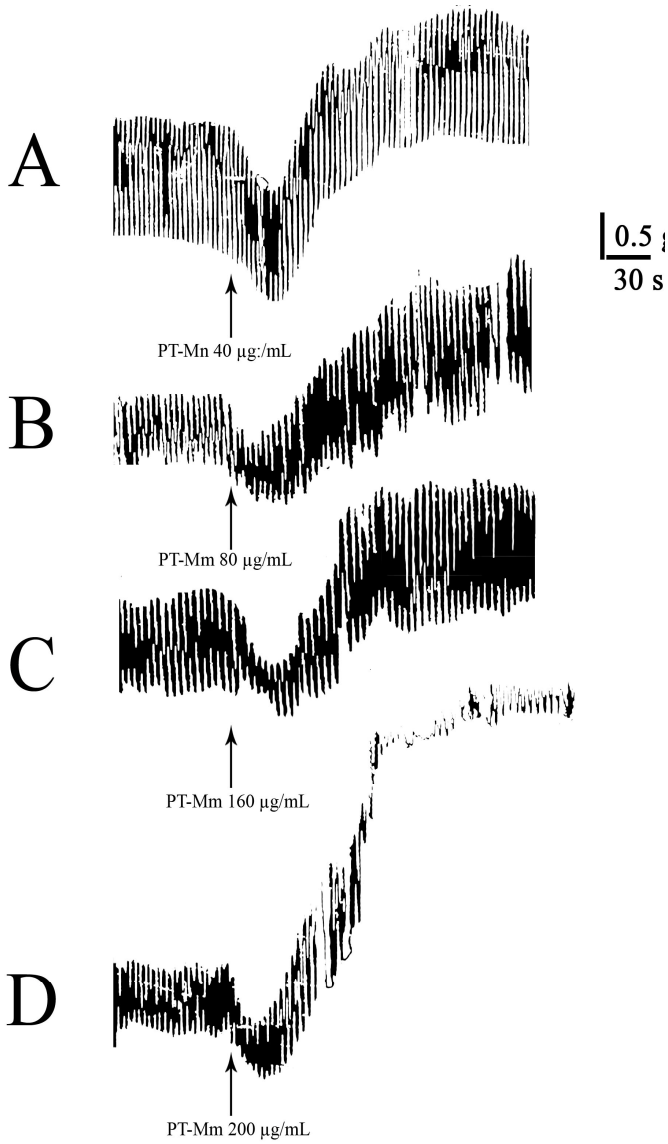

Figure 1: Dose-response effect of PT-Mm rabbit duodenum.

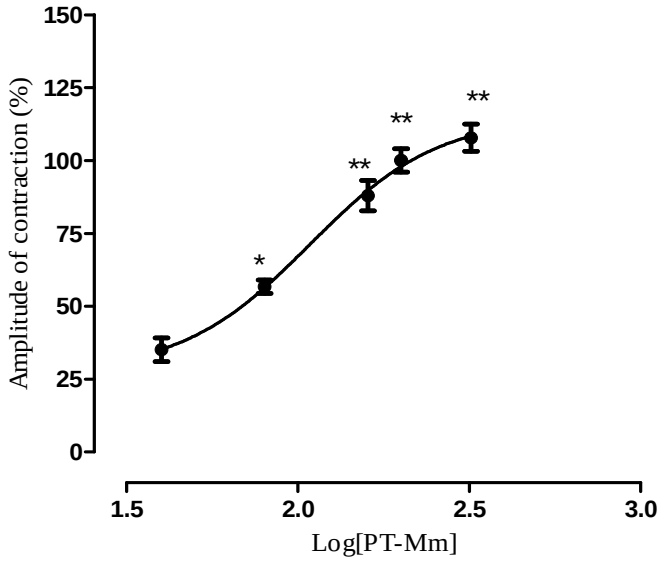

Figure 2: Dose-response curve of graded concentrations of PT-Mm extract on rabbit isolated duodenum. Values are expressed as mean ± S.E.M (n = 3). * P < 0.05; ** p < 0.01 compared to control.

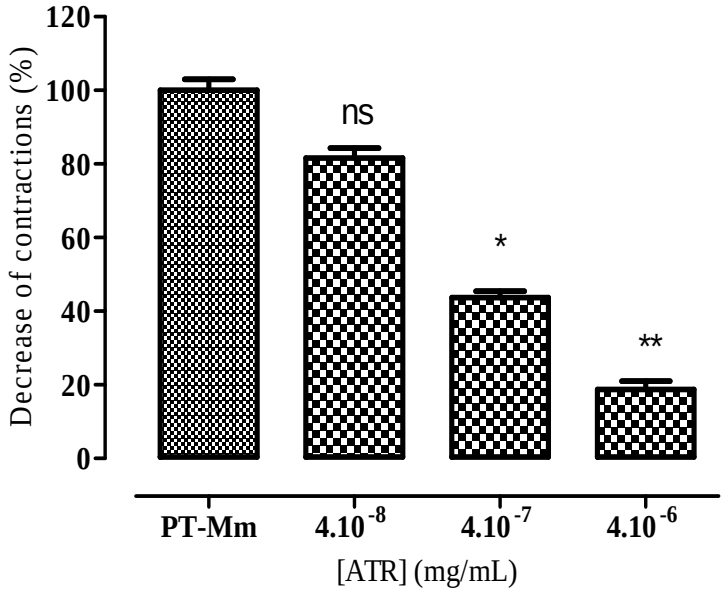

Figure 3: Antagonistic effect of Atropine and PT-Mm extract on the duodenal contractions. Values are expressed as mean ± S.E.M (n = 3) * P < 0.05; **P < 0.01 compared to control.

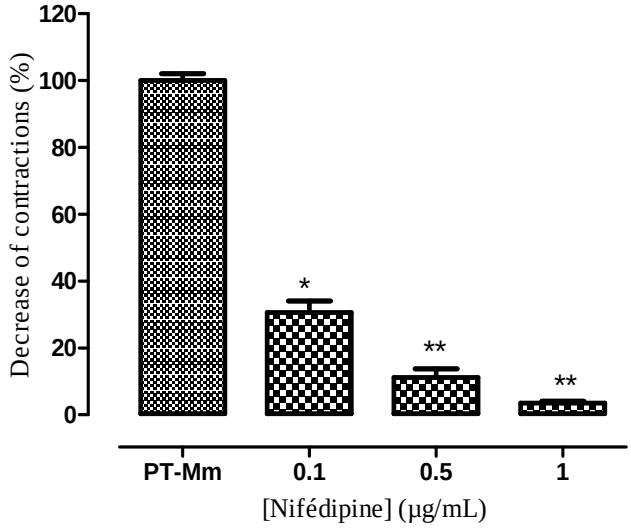

Figure 4: Antagonistic effect of Nifedipine and PT-Mm on the duodenal contractions. Values are expressed as mean ± S.E.M (n = 3). * P < 0.05; **P < 0.01 compared to control.

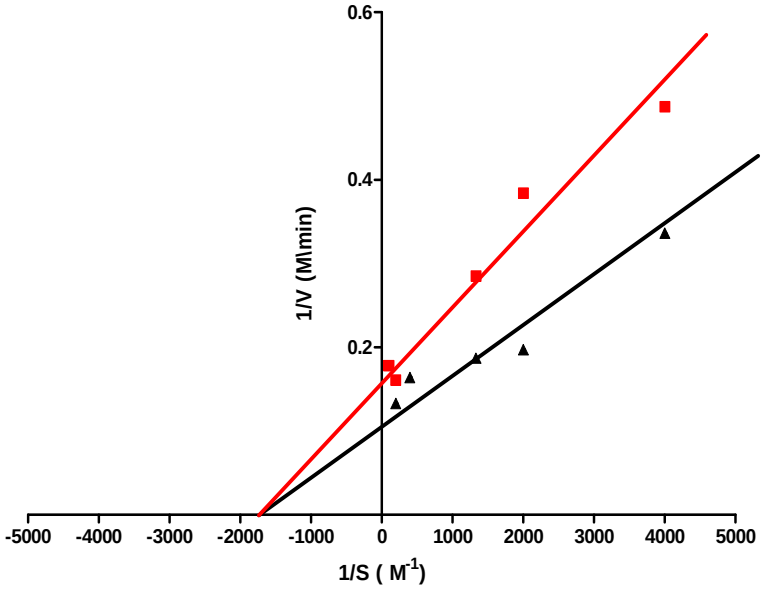

Figure 5: Linewaver-Burk (LB) plot of initial enzyme velocity (V) against the acétylthiocholine iodode concentration ([S]) in the presence (▲) and absence (■) of PT-Mm.

DISCUSSION

The leaves *Morinda morindoïdes* are usually used in folk medicine for the treatment of gastro-intestinal disorders. On the one hand, the pharmacological study of PT-Mm on isolated rabbit duodenum revealed significant and dose-dependent increase in tonicity and amplitude of the spontaneous contractions for varying concentrations (40 and 200 µg/mL). Similar results were demonstrated with *Harpagophytum procumbens* on smooth muscle preparations (Mahomed et al., 2005). Furthermore, it is well documented that acetylcholine (ACh) produces spasmogenic effect (Nene-Bi et al., 2009) as PT-Mm on the same muscle. ACh is known to induce contraction by the activation of muscarinic receptors (Naseri and Heidari, 2007) which in turn, increases the intracellular calcium though inositol triphosphate (IP_3) (Naseri and Heidari, 2007) and also by facilitating the inflow of extracellular calcium though the receptor-operated calcium channel (Zang et al., 2005). The fact that PT-Mm presented myostimulant action as Ach, allowed us to suggest that the plant extract may be contained cholinergic active constituents which would explain its use in the treatment of intestinal diseases.

On the other hand, our results have indicated that, the different concentrations of atropine (an inhibitor of the muscarinic receptors) reduced significantly (P□ 0.05) the increasing effect of the extract by the decrease of the amplitudes of contraction. Atropine blocked the activation of muscarinic receptors which caused the muscle contraction. This antagonistic test shows the existence of cholinomimetic compounds and precisely muscarinic receptors which are responsible for myostimulant effects in PT-Mm extract. These observations are in concordance with the findings of Goueh et al. (2009) and Meite et al. (2010) on the extracts of and *Trema guineensis and Mareya micrantha* respectively.

This study had also shown that nifedipine completely inhibited (p□ 0.01) the effect of extract by its calcium channels blocking action. We can say that, when nifedipine blocks calcium channels, the effect of plant extract is inhibited because the smooth muscle contraction (including rabbit duodenum), essentially depends an increase in the cytoplasmic free calcium, which activates the contractile elements. Furthermore the increase in intracellular calcium is due to either influx via voltage-dependent calcium channels or to release from intracellular stores in the sarcoplasmic reticulum (Bashir et al., 2011). Thus, PT-Mm induced its effect though opening calcium channels. This result is similar with the works of Gilani et al. (2007) on *Saussured lappa*.

In addition to its cholinergic effect, this study demonstrates the inhibition of AChE by PT-Mm. This anticholinesterase activity of PT-Mm corresponds to duodenal contraction. It should be noted that AChE has several peripheral anomeric catalytic sites (Johnson and Moore, 2006).These peripheral sites include the accelerating sites which would be the sites of binding of activators and inhibiting sites which would bind the inhibiting compounds. PT-Mm exerted a non-competitive inhibiting effect on AChE. These cholinomimetic and anticholinesterase effects are comparable with those of prostigmine and neostigmine which, by inhibiting AChE, increase the peristaltic movement of the intestinal smooth muscle (Krakowsky et al., 1997). This observation agrees with earlier reports (Mahomed et al., 2005; Dodehe et al., 2010) on some biological activities of *Harpagophytum procumbens* and *Combretum molle* respectively.

Conclusion

The results of this work revealed that PT-Mm contains pharmacologically active molecules which exert cholinomimetic and anticholinesterasic effects on duodenal smooth muscle. This physiological activity could support the use PT-Mm extract of *Morinda morindoïdes* in the treatment of intestinal disorder. Further research needed to

fractionate PT-Mm extract and isolate the molecule(s) responsible for the spasmogenic activity observed.

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