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Research Article

In vitro anticholinesterase and cholinergic effect of the aqueous extract of *Trema guineensis* on rabbit duodenum

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Abstract

Purpose: *In previous studies, the aqueous extract of Trema guineensis (Ulmaceae) was shown to increase rabbit duodenal contractions. The aim of this study was to evaluate the mechanism of the stimulation of the rabbit duodenum by the aqueous extract of Trema guineensis (Ulmaceae).*
Methods: *The in vitro organ bath method was used in our experiments. The enzyme was extracted from rabbit duodenum. The acetylcholinesterase (AChE) activity was determined by Ellman's assay using acethylthiocholine iodide as substrate. The rate of hydrolysis of acetylcholinesterase was monitored at 412 nm using a spectrophotometer.*
Results: *The effective concentration which induces 50% effect of Hypo+ (EC$_{50}$) was obtained with 8 x 10^{-2} mg/ml of Hypo+. The tests carried out in the presence of atropine showed a considerable reduction in the amplitudes of intestinal contractions. Hypo+ exerted mixed competitive inhibition on acetylcholinesterase (Vmax = 8.33 µM/min and K$_M$= 6.25 x 10^{-4} M). These results indicate that the crude extract of Trema guineensis contains anti-AChE and cholinomimetic substances. These two properties can explain the increase of duodenal contraction by Hypo+.*
Conclusion: *These results support the use of Trema guineensis as a laxative due to its stimulating effects on duodenal contractility.*

Key words: Trema guineensis, Myostimulation, Acetylcholinesterase, Hypo+, Duodenal contraction

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INTRODUCTION

The importance of the African traditional medicine in the management of diseases has long been established[1]. In Ivory Coast, traditional medicines are increasingly sought from tradipractitioners and herbalists for the treatment of various diseases. Among the remedies used, plant drugs constitute an important part. A number of scientific investigations have highlighted the importance and the contribution of many medicinal plants[2].

Trema guineensis (Ulmaceae) is a woody plant distributed in the west central part of Ivory Coast. The leaves are locally used for the treatment of various maladies including cardiac failure and constipation[3]. Previous studies (unpublished) have indicated that the aqueous extract of *Trema guineensis* induced the stimulation of the rabbit duodenum.

In an effort to elucidate the mechanism of duodenal stimulation by *Trema guineensis*, we have studied the antagonism between its aqueous extract and atropine. Furthermore, the effects of the aqueous extract have been tested against the catalytic activity of the acetylcholinesterase extracted from the duodenal muscle.

MATERIALS AND METHODS

Plant material

The leaves of *Trema guineensis* were collected in Daloa (west central region of Ivory Coast), in March 2004. The botanical identification of the plant was done by the herbarium of Centre National de Floristique, Abidjan, where a voucher specimen was conserved with reference number 13968.

Chemicals

All chemicals were of analytical grade and obtained from Sigma Chemical Co. (St Louis, MQ, USA), Aldrich Chemical Co. (Steineheim, Germany), and Merck (Darmstadt, Germany).

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, Ivory Coast, were used for the experiments. All the animals were housed at constant humidity (60%) and temperature (25°C) in a 12-hour light/dark cycle. The animals were cared for and treated according to the principles for the care and use of laboratory animals, and approval for the studies was given by the Ethical Committee of the University of Cocody-Abidjan.

Preparation of the aqueous extract

The freshly collected leaves of the plant were air-dried at room temperature for 7 days and powdered. Briefly, 200 g of powder was soaked in 2 L of distilled water for 24 h with constant stirring at 100 °C. The suspension was filtered through Whatmann (N°1) filter paper and the filtrate was concentrated *in vacuo* using a rotary evaporator to obtain the dry aqueous extract.

Preparation of isolated duodenal strips

After 24 hours of fasting, the animals were anaesthetised, killed and a median laparotomy was carried out. The 10 cm nearest to the gastroduodenal junction was discarded. Duodenal muscle strips (25 to 30 cm), free from adhering tissues, were removed and set up for recording isotonic contractions in 100 mL jacketed organ bath containing Tyrode solution (in mM : NaCl 130.5, KCl 5.63, $CaCl_2$ 2.16, $MgCl_2$ 0.24, NaH_2PO_4 1.18, $NaHCO_3$ 11.90, glucose 11.10) at 37°C saturated with pneumoxide (95 % O_2 + 5 % CO_2) and maintained at pH 7.8 ± 0.1. The duodenal strips were mounted in 100 ml jacketed tissue baths by suspending them between two L-shaped stainless steel hooks.

Measurement of isometric tension

The *in vitro* organ bath method[4] was used in our experiments. The tissue strips were initially set to 4 g tension (30 min loading

phase). After this period, the tension in each strip was readjusted to a baseline of 2 g (30 min adaptation phase). After this equilibration period, spontaneous contractions were recorded for 5 min in the absence (i.e., control) or presence of increasing doses (8×10^{-3} mg/ml to 4×10^{-1} mg/ml) of the aqueous extract. To study the effect of the aqeuous extract of *Trema guineensis* (Hypo+) on cholinergic transmission, spontaneous contractions were measured in the presence of atropine (a muscarinic cholinergic antagonist). After a dose, the strip was scoured four times (immediately, after 5 min and later twice after 10 min on each occasion) for complete removal of contractile compounds and recovery of the strips. The effect of each dose was calculated as a percentage of the maximal increase of contraction (100 %) obtained with Hypo+ (0.4 mg/ml).

Enzyme assay

Enzyme extraction was performed according to the method of Khoa and Ochillo[5]. A length of duodenum weighing 1g was added to 50 ml phosphate buffer and crushed with a mortar (Ultra Turax T25). The homogenate was centrifugated and the supernatant was used for the assays. Acetylcholinesterase activity was determined spectrophotometrically by Ellman's assay[6]. To each cuvette was added 5,5'- dithio-bis-(2-nitro) benzoic acid (DTNB) (100 µL of 0.01 M DTNB in 50 mM potassium phosphate buffer, pH 7.8) followed by the addition of ATCh (25 µL of ATCh of varying concentration in 50 mM potassium phosphate buffer, pH 7.8). The enzymatic reaction was initiated at 25 °C by the addition of enzyme (75 µL of homogenate, appropriately diluted in 50 mM potassium phosphate buffer, pH 7.8), and the absorbance change was monitored at 412 nm with a spectrophotometer (Alresa, Barcelona, Spain).

Enzyme kinetic analysis

To determine the enzyme kinetics of the aqueous extract of Hypo+, the kinetic analysis of the duodenum AChE solution in the presence of the extract was performed. The mixture of enzyme and Hypo+ was incubated 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 Hypo+ was determined by the Lineweaver-Burk (LB) plot. The LB plot represents reciprocal velocities *vs.* the reciprocal substrate concentration of the control (without inhibitor) and the series of inhibitor concentrations[7].

Statistical analysis

Data were analyzed by one-way ANOVA followed by Dennelt's t-test using computerized Graph Pad and $P < 0.05$ was considered statistically significant.

RESULTS

Dose-response effect of Hypo+ on rabbit duodenum

Figure 1 represents the recordings of the mechanical activity of the rabbit duodenum in the presence of Hypo+ for concentrations ranging from 8×10^{-3} to 4.10^{-1} mg/ml. These recordings were obtained on the same preparation. The perfusion of 8.10^{-3} mg/ml of Hypo+ increased the amplitude of contraction by 14.28 ± 2.8 % (Figure 1A) of the maximal increase in amplitude (100 %). At 8.10^{-2} mg/ml of Hypo+, the increase in amplitude of duodenal contractions was 42.85 ± 3.5 % (Figure 1B) while at 1.6×10^{-1} mg/ml of Hypo+, the increase in amplitude rose to 71.42 ± 4.2 % (Figure 1 C). With 2.4×10^{-1} mg/ml of Hypo+, the amplitude of contraction indicates 85.71 ± 5 % of the maximum recorded (Figure 1D) while at 4×10^{-1} mg/ml of Hypo+, the amplitude of contraction reached its maximum (100 %). Figure 2 expresses the
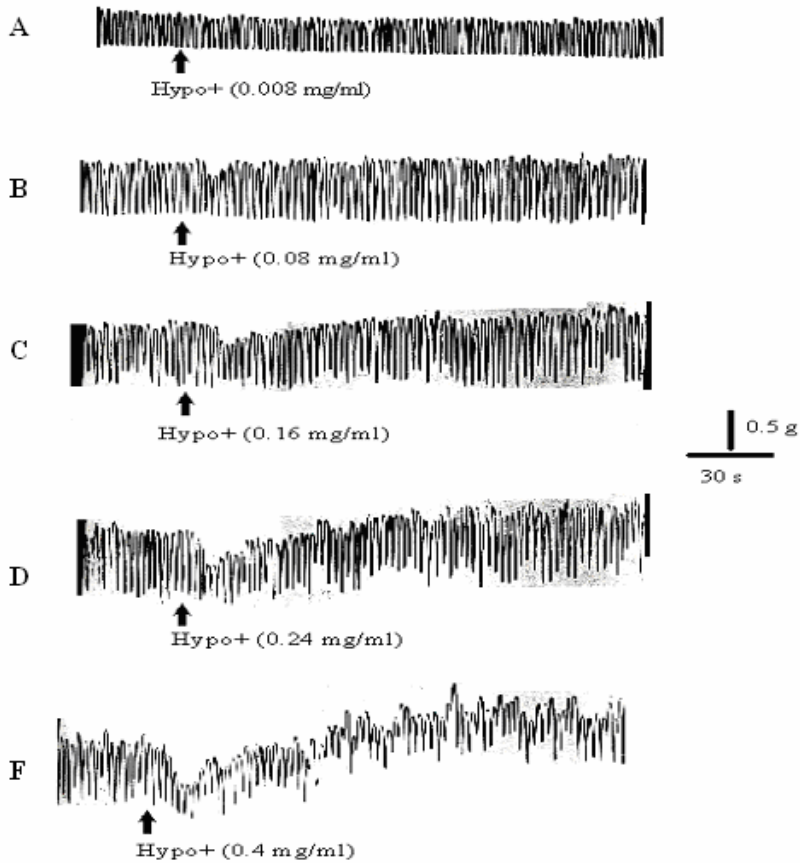
Figure 1: Dose response-effect of Hypo+ on the duodenal contraction
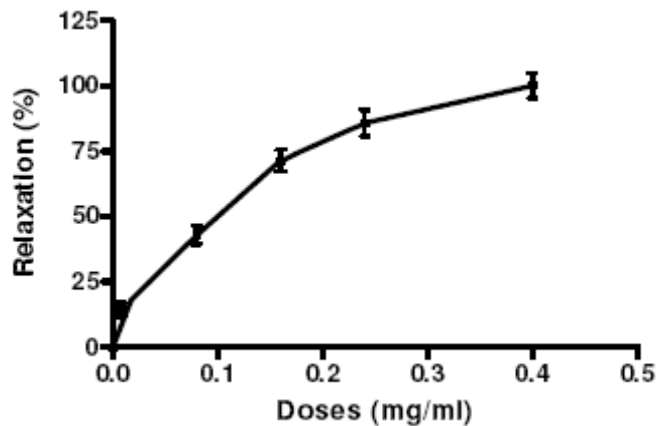

Figure 2: Dose response curve showing the duodenum relaxation induced by increasing doses of Hypo+. The values were expressed as the mean ± SD (n=4)

mean (N = 4) of the maximum variation of the amplitude of rhythmic contractions of the duodenal strips against the dose of Hypo+. EC_{50} (effective concentration which induces 50% effect of Hypo+) was determined from the curve of the amplitudes to be 8 x 10^{-2} mg/ml.

Antagonistic effects of atropine and Hypo+ on duodenal contractility

The recording in Figure 3 represents the interaction between Hypo+ (0.4 mg/ml) and the various increasing concentrations of atropine. After the perfusion of Hypo+ at a dose of 0.4 mg/ml in the tank containing 0.04 ng/ml of atropine (Figure 3A), the amplitude of contraction increased slightly (27.5 ± 4.1 %) compared to the control (Figure 1E). At a dose of 0.4 mg/ml of Hypo+ in the presence of atropine (0.4 ng/ml), the increase in amplitude of contraction was low (15 ± 4.8 %) compared to the control (Figure 3B). Finally, at a dose of

0.4 mg/ml of Hypo+ in the presence of atropine (4 ng/ml) the amplitude of contraction did not vary any further (figure 3C). The contraction of the duodenum was blocked by atropine at this dose.

Effect of Hypo+ on the hydrolytic action of AChE

The kinetic analysis of AChE inhibition by Hypo+ (20 mg/ml) is shown in Figure 4. Hypo+ inhibited AChE in a non-competitive manner. The chart of Lineweaver and Burk[8] in which the lines obtained cross the y-axis and that of 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 Hypo+ were 5 µM and 81.6 µM/min, respectively. In the presence of Hypo+, the corresponding values are : K_M (5 µM) and Vmax (46 nM/min).
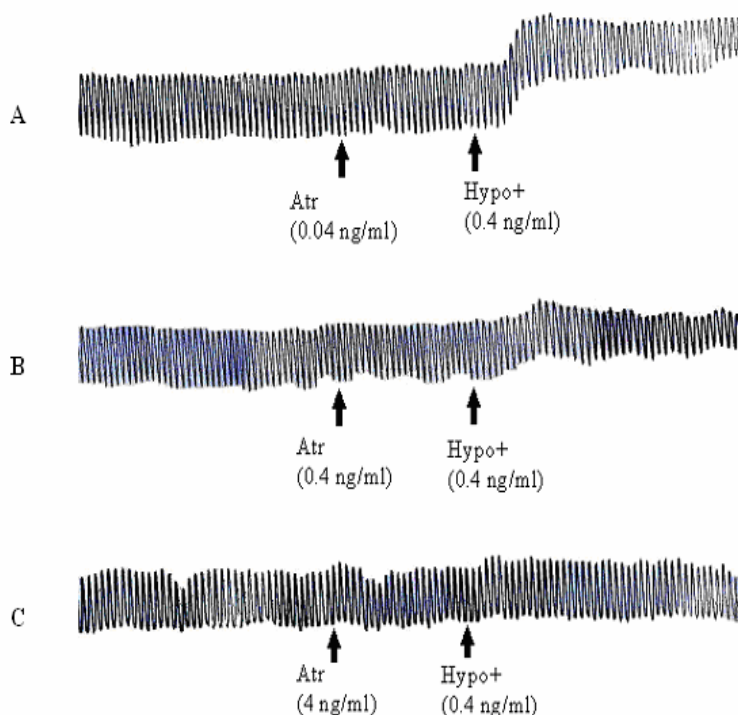

Figure 3: Antagonistic effect of atropine and Hypo+ on duodenal contraction
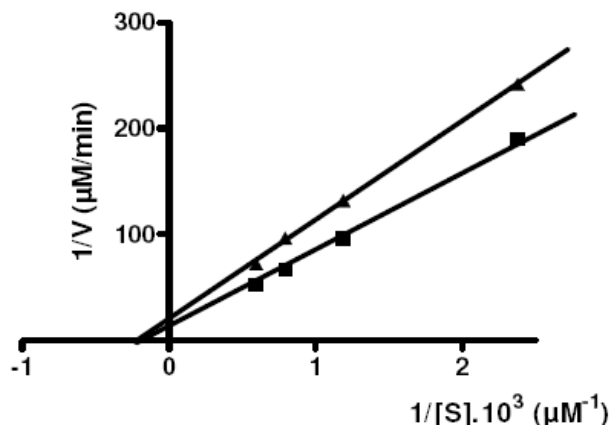
Figure 4: Lineweaver-Burk (LB) plot of initial enzyme velocity (V) against the acetylthiocholine iodode concentration ([S]) in the presence (▲) and absence (▪) of Hypo+

DISCUSSION

The effect of Hypo+ on duodenual contractility are similar to those of acetylcholine on the same muscle. It is well documented that acetylcholine produces contractile effects on smooth muscle[9]. Our results show that Hypo+ increases the spontaneous and tonic contractions of rabbit duodenum. These contractions, initiated by Hypo+, are reduced under the effect of atropine. The spontaneous movements of the duodenum are regulated by cycles of depolarization and repolarization[9]. According to Rodger[10], ACh induces tonic contractions by depolarization and by mobilizing the extra-cellular Ca^{2+} in the cytosol along the calcium channels. Any substance which shows cholinomimetic action and raises the amplitude of gastro-intestinal movements can be classified as diarrhoeal-inducing[11] because they stimulate the contraction of intestinal muscular tissue. Moreover, atropine reduces to a significant degree the stimulating effect of Hypo+. This observation suggests that Hypo+ may contain cholinomimetic substances which would be bound to the muscarinic receivers.

In addition to its cholinergic effect, this study demonstrates the inhibition of AChE by the aqueous extract of *Trema guineensis.* The inhibition of AChE by the aqueous extract of *Trema guineensis* is, to the best of our knowledge, reported in this study for the first time. This anticholinesterase activity of Hypo+ corresponds to duodenal relaxation.

It should be noted that AChE has several peripheral anomeric catalytic sites[12,13,14]. These peripheral sites include the 'accelerating' sites which would be the sites of binding of activators and the 'inhibiting' sites which would bind the inhibiting compounds. The aqueous crude extract of *Trema guineensis* exerted a non-competitive inhibitory 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[15].

CONCLUSION

This work was designed to study the mechanism of the stimulation effect of Hypo+ on duodenum contractility. Hypo+ exerts cholinimimetic and anticholinesterasic effects which are in concordance with the physiological effects of the aqueous extract of *Trema guineensis* and can support the use of this plant as laxative in traditional medicine.

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