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(RESEARCH ARTICLE)

Anticholinergic and adrenergic activities of a dichloromethane fraction of a crude ethanol extract of stem bark of *Piliostigma reticulatum* Horscht D.C (Ceasalpiniaceae)

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Abstract

The objective of the present study was to investigate the anticholinergic and adrenergic activities of a dichloromethane fraction of a crude ethanol extract of stem bark of *P. reticulatum,* on isolated rabbit duodenum contractions. In the first study, the dichloromethane fraction was tested on the isolated rabbit duodenum contractions at increasing concentrations (0.13; 0.26; 0.39 and 0.52 mg/mL) after addition of acetylcholine. In the second study, the dichloromethane fraction was tested on the isolated rabbit duodenum contractions ten minutes after addition of prazosin or propanolol at increasing concentrations (10^{-4}, 10^{-3} and 10^{-2} mg/mL). The amplitude and tone induced by acetylcholine were significantly inhibited by the dichloromethane fraction. At a concentration of 0.52 mg/mL, the amplitude induced by the acetylcholine was 0.42 ± 0.05g. The relaxation induced by the dichloromethane fraction was significantly inhibited by prazosin and propanolol as well. At a concentration of 10^{-2} mg/mL, prazosin and propanolol inhibited the relaxation induced by the dichloromethane fraction at rates of 18.6 ± 2.42 and 17.4 ± 0.7 % respectively. Results showed that the dichloromethane fraction of crude ethanol extract of stem bark of *Piliostigma reticulatum* exhibited anticholinergic and adrenergic properties that could at least explain and back up its traditional use against diarrhea.

Keywords: Acetylcholine; Diarrhea; *Piliostigma reticulatum*; Prazosin; Propranolol**.**

1. Introduction

Today the treatment of spastic motility disorders is a real concern. Therapeutic options remain limited on account of our failure to understand pathophysiology and its significance [1]. Drugs are used against hyper-contractility of smooth muscles and allow the gastrointestinal muscle to return to their proper tone [2]. Antispasmodic drugs include anti-muscarinic compounds (e.g. Alkaloids derived from belladonna and their synthetic derivatives) and calcium channel blockers (e.g. otilonium and pinaverium) are the principal modern drugs used by population to treat gastrointestinal disorders [3, 4]. These drugs in addition to their pharmacological effects are responsible of some adverse effects in the organism.

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Thus, some medicinal plant species are used in traditional medicine by rural populations for the treatment of gastrointestinal disorders including diarrhea, indigestion and constipation [5, 6, 7]. In order to determine the mechanism of these plants in the treatment of gastro-intestinal disorders, scientists use essentially the antagonism study on the intestine contraction with chemicals products like acetylcholine, prazosin and propranolol. Studies carried on some isolated organs demonstrate that some medicinal plant species have anticholinergic or adrenergic effect [8, 9, 10].

Piliostigma reticulatum used in this study, is found in the tropical forests of west african countries like Côte d'Ivoire, Mali and Burkina Faso and is traditionally used against ailments such as ulcers [11], boils, wounds, syphilitic cancer [12, 13] and diarrhea [12, 14, 15]. In our previous studies, before toxicity study [16], the dichloromethane fraction showed an activity against diarrhea [17], electrolytes secretion [18] and bacterial diarrhea [19]. The effects of *Piliostigma reticulatum* are studied on biochemical parameters in rats [20]. Therefore, the mechanism of the spasmodic property of this plant has not yet been investigated to our knowledge. The present work was designed to assess the anticholinergic and adrenergic effects of the dichloromethane fraction on contractions of smooth muscle of an isolated rabbit duodenum.

2. Material and methods

2.1. Plant material

Stem barks of *Piliostigma reticulatum* (DC.) Horscht (Ceasalpiniaceae) were collected in Abidjan (South region of Côte d'Ivoire). The plant was identified and authenticated by the National Centre of Floristic of the University of Félix Houphouet Boigny-Abidjan by Pr AKE ASSI. A voucher specimen (N° 18033) of the plant was deposited in the herbarium of the National Centre of Floristic of the University of Félix Houphouet Boigny- Abidjan.

2.2. Preparation of the ethanol extract and dichloromethane fraction

Stem barks of *Piliostigma reticulatum* were washed with distilled water, cleaned, cut into small pieces and kept at room temperature for two weeks. Then, they were ground into a fine powder. The powder (100 g) was suspended in 2 liters of a solution of ethanol (96%) / water (80:20) for 24 hours under constant stirring (this operation was repeated twice). The extract was filtered twice through cotton wool, then through Whattman filter paper (N° 1). The filtrate was evaporated to dryness using a rotavapor (Buchi R110/NKE6540/2), and dried under reduced pressure. Five liquid fractions (heptane, dichloromethane, ethyl acetate, butanol and aqueous) were obtained from the crude ethanol extract by using successive liquid–liquid extraction [21, 22]. The dichloromethane fraction was used because of its efficiency in preliminary studies compared to the others.

2.3. Animals

Healthy rabbits (weighing 1.5-2 kg) provided by the faculty of Biosciences (University of Félix Houphouet boigny - Abidjan, Côte d'Ivoire) were used. They were kept and maintained under standard laboratory conditions one week prior to experiments. Animals were fed with food pellet (Ivograin®, Abidjan, Côte d'Ivoire) and were given water *ad libitum*. They were deprived from food and for at least 24h prior to experiments.

Experiments were carried out in accordance with the European Council legislation 87/609/EEC for the protection of experimental animals [23].

2.4. Smooth muscle preparation

The experiments were carried out according to the general technique of Magnus [24]. The animals were killed by median laparotomy and the duodenum was removed. Two (2) cm long of the duodenum segment was suspended in 150 mL organ bath containing Tyrode's solution of the following composition (mM): NaCl 136.89, KCl 2.68, $CaCl_2$ 1.80, $MgCl_2$ 1.05, $NaHCO_3$ 11.90, $NaHPO_4$ 0.42 and glucose 5.55, maintained at 37 °C. The solution was aerated with a mixture of 95% O_2 and 5% CO_2.under a resting tension of 1g. The preparations were connected to a transducer coupled to the paper graph. The suspended duodenum was allowed to equilibrate for 30 min. The smooth muscles relaxant action of test materials was observed by administration of extracts [25]. The bath was washed after testing each concentration of the extract.

The inhibition of duodenum contraction by test sample was expressed as mean percentage ± SEM from six experiments in the presence of the extract and was calculated using the following formula:

$$\%\ Inhibition = \frac{A - B}{A} \times 100$$

Where A is the amplitude (cm) of the normal duodenum contraction and B the amplitude (cm) of the duodenum contraction induced by the extract in the presence of the test sample [26].

2.5. Study of antagonism

For the antagonism between acetylcholine and the dichloromethane fraction, 7.09 10^{-4} mg/mL of acetylcholine were tested on intestine contraction. This concentration of acetylcholine was the IC_{50}, obtained after the dose-response study. The concentrations of 0.13; 0.26; 0.39 and 0.52 mg/mL were successively tested just after the acetylcholine effect on intestine contraction [27]. Prazosin and propanolol were tested on intestine contraction at concentrations of 10^{-4}, 10^{-3} and 10^{-2} mg/mL. The concentration of 0.24 mg/mL of dichloromethane fraction which was the IC_{50} after the dose-response study was tested 10 minutes after prazosin or propanolol effect on the intestine contraction [28].

2.6. Source of chemical products

Acetylcholine, prazosin and propanolol were collected from SIGMA (St. Louis, MO, USA).

2.7. Phytochemical screening

The dichloromethane fraction was screened for the presence of tannins, flavonoids, alkaloids, sterols, saponins, polyphenols, polyterpenes and quinons. Detection of these phytocompounds was performed as described by [29].

2.8. Statistical analysis

The results were expressed as mean ± SEM. Data were analyzed for statistical significance by one-way ANOVA followed by Tukey post-hoc test using the software GraphPad prism (San Diego) 5. $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Extraction of plant material

The amount of total ethanol extract was 13.6%. From a dried total ethanol extract (10 g), we successively obtained heptane fraction (90 mg; 3.6%), dichloromethane fraction (200 mg; 8%), ethyl acetate fraction (500 mg; 20%), butanol fraction (700 mg; 28%) and aqueous fraction (900 mg; 36%).

3.2. Anticholinergic activity of dichloromethane fraction

The introduction of dichloromethane fraction at concentrations of 0.13; 0.26; 0.39 and 0.52 mg/mL provoked the inhibition of amplitude and tone induced by acetylcholine (figure 1).

Decrease of contraction amplitude induced by acetylcholine was significant ($p < 0.01$) and were 4.7 ± 2.2; 3.3 ± 1.9; 1.12 ± 0.8 and 0.42 ± 0.05g respectively at concentrations of 0.13; 0.26 ; 0.39 and 0.52 mg/mL for dichloromethane fraction (figure 2). The dichloromethane fraction significantly decreased ($p < 0.01$) the tone induced by acetylcholine to 0.08 ± 0.01g at the concentration of 0.52 mg/mL (figure 3).

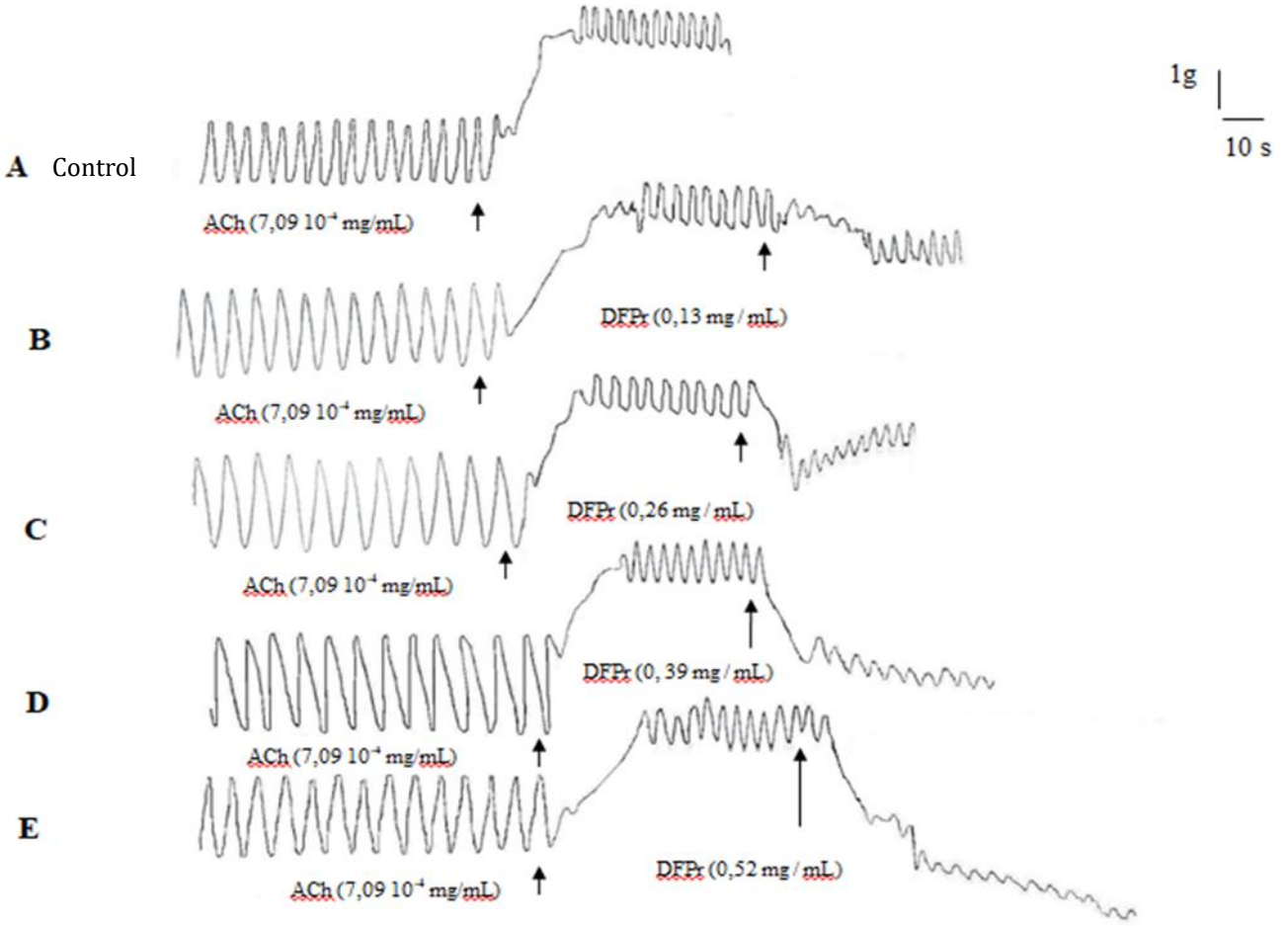

Figure 1 Interaction ACh-Dichloromethane fraction (DFPr) on contractions of isolated rabbit duodenum (n = 6).

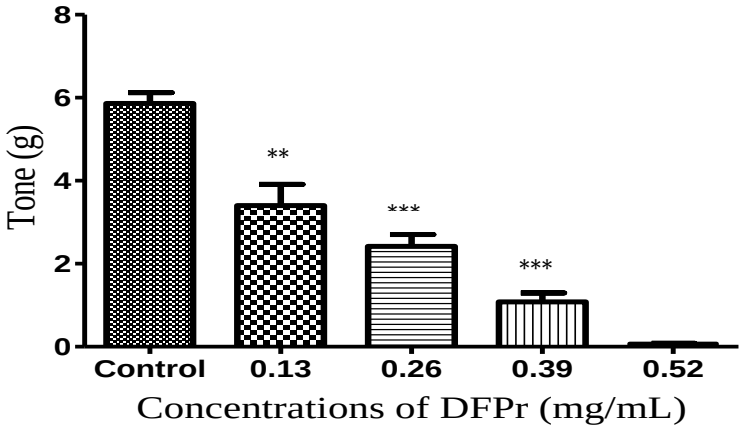

Figure 2 Decrease of the amplitude of intestine contractions induced by ACh in presence of DFPr (**P < 0.01; ***P < 0. 001; n = 6).

DFPr = Dichloromethane fraction of *Piliostigma reticulatum*

ACh = Acetylcholine

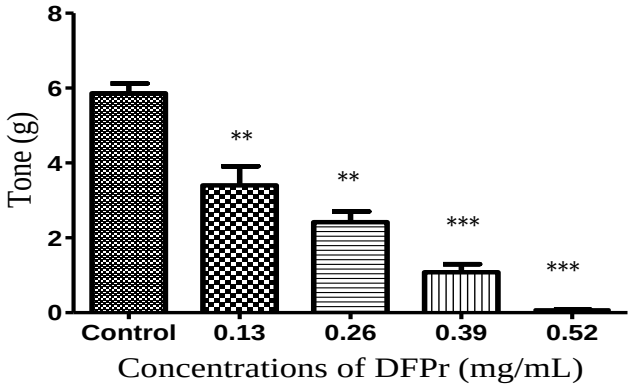

Figure 3 Decrease of tone of intestine contractions induced by ACh

in presence of DFPr (***P < 0. 001; n = 6).

DFPr = Dichloromethane fraction of *Piliostigma reticulatum*

ACh = Acetylcholine

3.3. Antagonism between prazosin (PRAZ) and dichloromethane fraction

The inhibition of amplitude decrease induced by dichloromethane fraction was correlated by the reduction or the relaxation percentage of the intestine smooth muscle. The percentages relaxation reduction were to 57.8 ± 1.07 % for 10^{-4} mg/mL and 18.6 ± 2.42 % for 10^{-2} mg/mL of PRAZ (figure 4). The reduction of the intestine muscle relaxation induced by the dichloromethane fraction was significant in presence of PRAZ (p < 0,001) (figure 5).

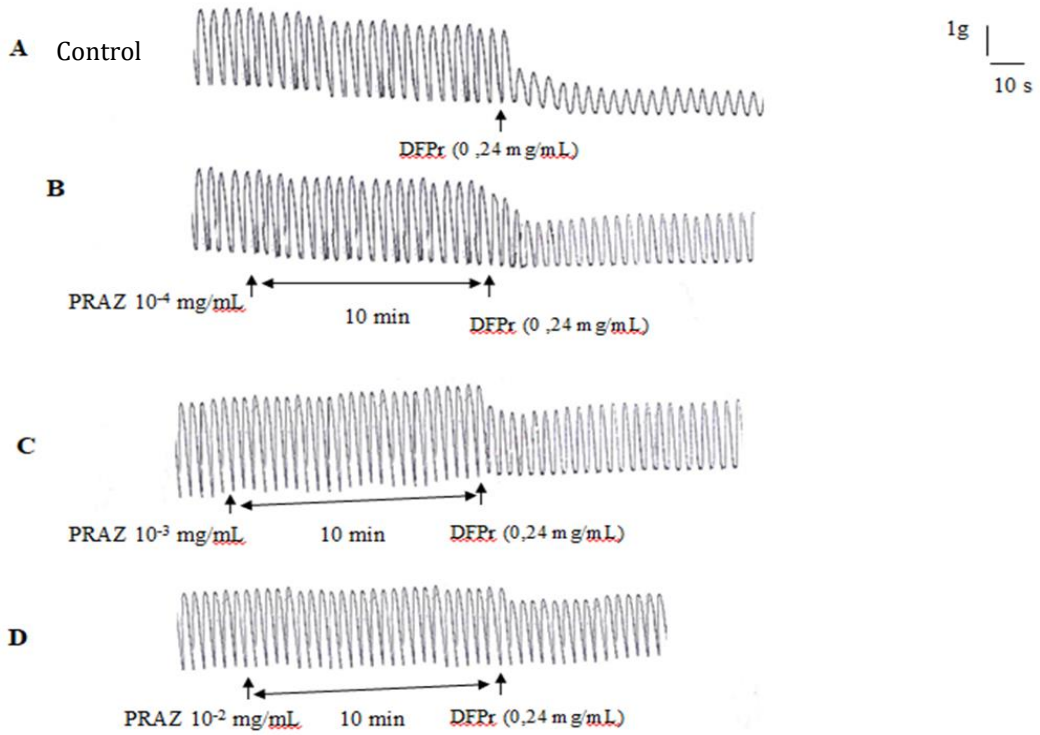

Figure 4 Interaction PRAZ-DFPr on contractions of isolated rabbit duodenum (n = 6).
DFPr = Dichloromethane fraction of *Piliostigma reticulatum* PRAZ = Prazosin

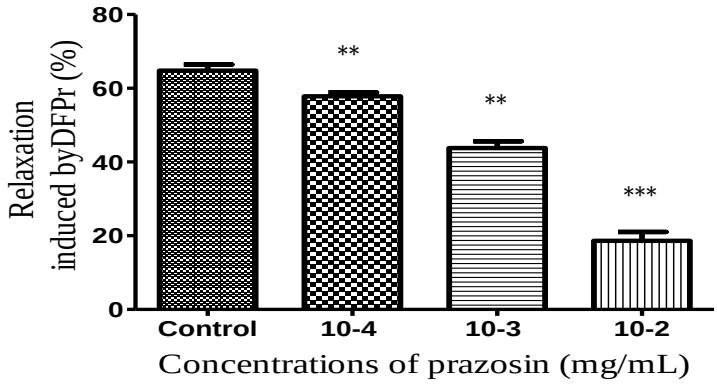

Figure 5 Effect of prazosin on relaxation induced by DFPr

(**p < 0.01; ***P < 0. 001; n = 6).

DFPr = Dichloromethane fraction of *Piliostigma reticulatum*

3.4. Antagonism between propanolol (PROP) and dichloromethane fraction

The percentages of reduction of contraction amplitude lowered to reach 52 ± 1.38; 35.6 ± 2.01 and 17.4 ± 0.7 % for dichloromethane fraction respectively at concentrations of 10^{-4},10^{-3} and 10^{-2} mg/mL for PROP (figure 6). The relaxation of the smooth muscle of duodenum was almost totally inhibited at the concentration of 10^{-2} mg/mL (figure 7). The inhibitions of relaxations were significant (p < 0,001) compared to those induced by the dichloromethane fraction at a dose of 0.24 mg/mL (figure 7).

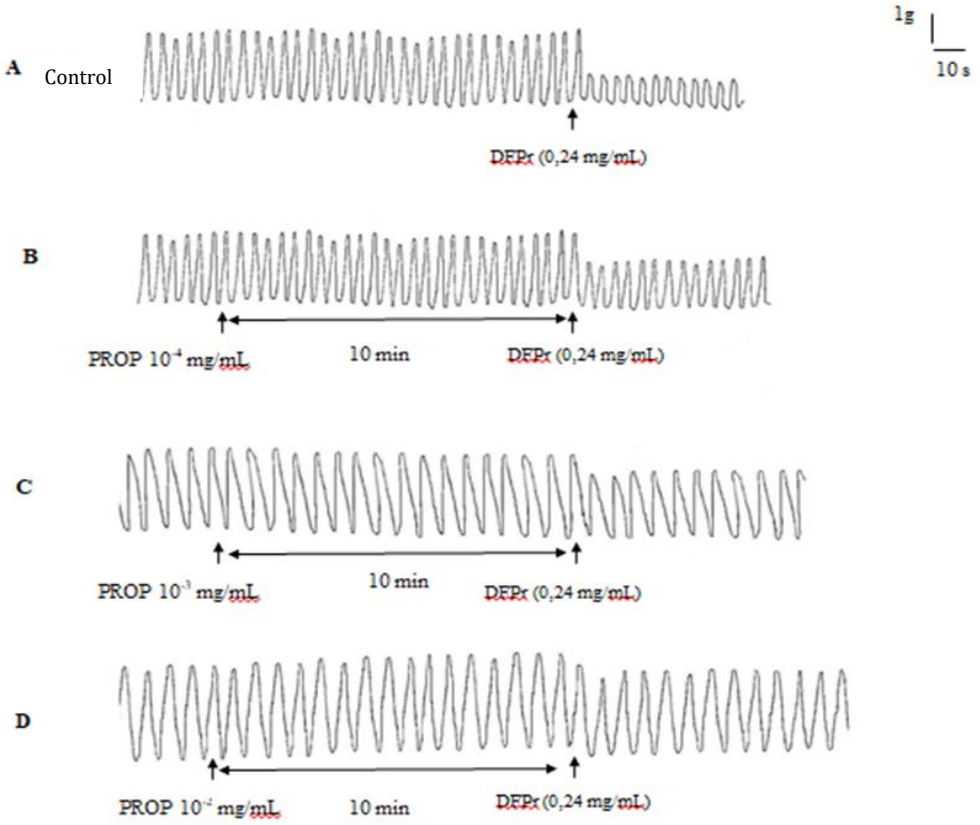

Figure 6 Interaction PROP-DFPr on contractions of isolated rabbit duodenum (n = 6).
DFPr = Dichloromethane fraction of *Piliostigma reticulatum* PROP = Propanolol

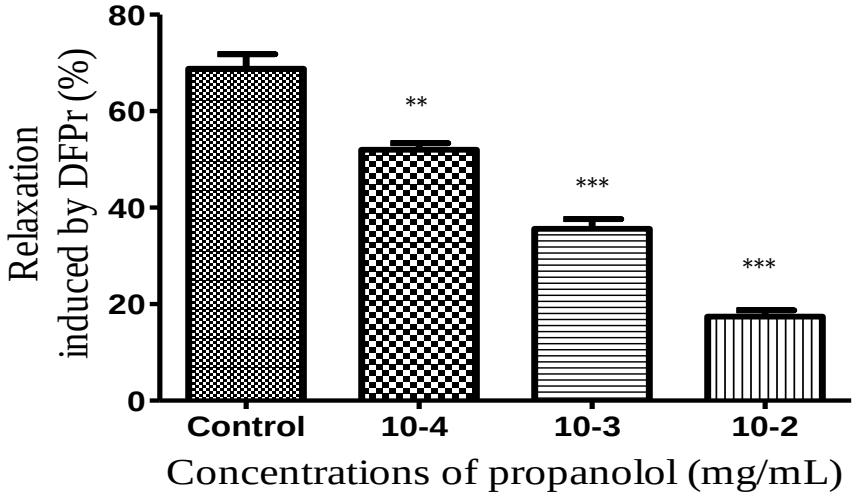

Figure 7 Effect of Propanolol on relaxation induced by DFPr
(**p < 0.01; ***P < 0. 001; n = 6).
DFPr = Dichloromethane fraction of *Piliostigma reticulatum*

3.5. Phytochemical tests

Phytochemical screening tests revealed the presence of tannins, flavonoïds, polyphenols and reducing sugars. However, Quinones, alkaloids, coumarins, polyterpenes and sterols were not found in dichloromethane fraction (table 1).

Table 1 Phytochemical screening of dichloromethane fraction of stem bark of *P. reticulatum*

chemical compounds	*Piliostigma reticulatutum*
Polyphenols	+
Sterols et polyterpernes	-
Flavonoids	+
Saponins	-
Tannins	+
Alkaloids	-
Quinones	-
Reducing sugars	+
Coumarins	-

(-) absence, (+) presence

4. Discussion

4.1. Anticholinergic activity of the dichloromethane fraction

These studies showed a significant reduction of negative inotrops and positive tonotrops effects induced by acetylcholine on intestine contractions. At a concentration of 0.52 mg/mL, dichloromethane fraction reduced to 11 ± 1.12 %, the percentage of amplitude contractions induced by acetylcholine. In addition, the tone induced by acetylcholine was significantly reduced by the dichloromethane fraction. This action of dichloromethane fraction on the effects induced by acetylcholine was dose-dependant. Our results are similar to those obtained by [9]. These authors demonstrated that the essential oil of *Monodora mystirica* leaves at a dose of 3µg/mL, totally inhibited contractions induced by acetylcholine. Furthermore, the inhibition of contractions induced by acetylcholine with species like Cymbopogon *giganteus* and *Salvadora persica* have been studied by [27]. Acetylcholine is a neurotransmitter liberated by parasympathic nervous system which plays a physiological key role in the regulation of intestine movements [30, 31]. Indeed, it is known that the effect of acetylcholine is translated by an increase of membrane permeability to calcium ions or by liberation of intracellular calcium due to the activation of muscarinic receptors of intestine muscle [32, 33].

According to the results above, the dichloromethane fraction could contain anticholinergic substances, responsible for the blocking of dichloromethane fraction fixation on muscarinic receptors. Consequently, this prevents the entry of extracellular calcium but the liberation of intracellular calcium. Many authors have shown these anticholinergic effects [34, 35, 36] with the aqueous leaves extract of *Cistus ladaniferus* (Cistaceae), the aqueous leaves extract of *Carum copticum* (Ombelliferous) and the ethanol leaves extract of *Helichrysum plicatum* (Asteraceae). The inhibition of intestine contractions induced by dichloromethane fraction could be responsible for slowing intestinal transit to inhibit diarrhea.

4.2. Interaction between dichloromethane fraction and prazosin or propanolol

These studies showed that the relaxation induced by the dichloromethane fraction is significantly inhibited by propranolol and prazosin. The abolition by propanolol and prasozin for the reduction of the amplitude of rythmic contractions of isolated rabbit duodenum observed with dichloromethane fraction could indicate the existence of adrenomimetic substances in this extract. These substances could operate on duodenum muscles through α and β-adrenergic receptors, respectively blocked by prazosin and propanolol [8, 28, 37]. In this case we can admit that the dichloromethane fraction could speed up α and β-adrénergic receptors. According to [38], this activation allows protein Gi ton inhibit the activity of adenylcyclase, provoking a falling of AMPc (Adenosin Monophosphate Cyclic) production. AMPc is not then able to speed up protein kinases which produce phosphate for regulating the cellular membrane proteins of the sarcoplasmic reticulum such as actins and myosins. The absence of phosphorylation provokes the closing of calcium canals, a deactivation of the sarcoplasmic reticulum calcic pomp and a falling of actins and myosins sensitivity for calcium. Thus, rhythmic contractions decrease. The dichloromethane fraction could operate by the same mechanism. Moreover, the dichloromethane fraction could contain adrenomimetic substances, responsible for intestine contractions inhibition.

Flavonoïds, tannins, polyphenols and reducing sugars in dichloromethane fraction could be responsible for the myorelaxant effects observed. Thus, tannins, flavonoïds and reducing sugars are generally responsible for myorelaxant properties of medicinal plants [39, 40]. Flavonoïds have already shown their ability to inhibit contractions induced by spasmogens [41]. The quercetol, which is a flavonoïd reduces the amplitudes of intestine contractions inhibiting acetylcholine secretion [39]. The anticholinergic and adrenergic effects observed with the dichloromethane fraction could be due to its chemical components.

5. Conclusion

The studies of interactions showed that the dichloromethane fraction inhibited intestine contractions induced by acetylcholine. Relaxations induced by dichloromethane fraction were inhibited by propanolol and prasozin, two chemicals blocking the action of adrenaline.

The diarrhea is often caused by a hypercontractility of the intestine; it is therefore probable that medicines that inhibit intestine contractions could be antidiarrheal compounds.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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