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Research article

**PRELIMINARY TEST FOR THE DETECTION AND CHARACTERIZATION OF
PHYTOMOLECULES RELAXING SMOOTH MUSCLE OF DICHLOROMETHANE-
ETHANOLIC EXTRACT OF *MORINDA MORINDOIDES* (BAKER) (RUBIACEAE)**

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ABSTRACT: The present study focused to investigate the detection and characterization of phytomolecules relaxing smooth muscle of dichloromethane-ethanolic extract of *Morinda morindoides* using *in vitro* model. Local rabbit of both sexes weighting between 1500 and 2000 g were used. The effect of the extract on contraction of isolated rabbit ileum and the response of the tissue was investigated. The extract at dose of 49×10^{-3} ; 190×10^{-3} ; 290×10^{-3} ; 390×10^{-3} mg/ml, attenuated significantly ($p < 0,05$) spontaneous contractions of the isolated rabbit ileum in a dose-dependant manner. The effective dose for 50% of activity was $193 \pm 1,5 \times 10^{-3}$. Tonic contractions initiated by acetylcholine were inhibited by the extract like atropine. The extract also attenuated the spontaneous contractions of the intestine in presence of propanolol. Phytochemical screening revealed the presence of sterols and terpenes, polyphenols, flavonoids, tannins, saponins, and alkaloids. This study provided the active compounds with relaxant activity of the extract would be cholinomimetic substances, and may possibly explain the use of the plant in traditional medicine for the treatment of gastrointestinal disorder.

Keywords: *Morinda morindoides*, cholinomimetic, rabbit ileum.

INTRODUCTION

Diarrhoea is a disorder characterized by discharge of semisolid or watery faecal matter from the bowel three or more times in a day (Mbagwu and Adeyemi, 2008). It has long been recognized as one of the most important health problems and leading cause of mortality and morbidity in the developing countries (Magaji, et. al., 2007, Rajamanickam, et. al., 2010) and produces more illness and causes death of more infants and children below 5 years old than all other diseases combined (Dalal, et. al., 2011) It is considered as one of the leading causes of growth retardation and death in infants (Petri, et. al., 2008). Diarrhoea was responsible for the death of millions people each year. Despite immense technological advancement in modern medicine, many people in developing countries still depend on traditional medicine for their primary healthcare needs (Ojewole, 2004). Use of traditional medicines to combat the consequences of diarrhea has been employed by WHO in its Diarrhea Control Programme (Syder, et. al., 1982, Lutterodt, et. al., 1982, Chitme, et. al., 2004, WHO, 2004 Atta and Mouneir, 2004 Sunilson, et. al., 2009). About 80% of people in developing countries use traditional medicines for their health care (Kim, 2005). Several plants have been reported to be used in treating and managing of diarrhoea (Agunu, et. al., 2005). One of such medicinal plants was *Morinda morindoides* (Baker) Milne-Redheat (Rubiaceae) a Guinean-Congolese species. The plant popularly called «Zèlékéle» in 'Bété' (a tribe in central part of Ivory Coast) is found widely in the borders of tropical forests (Méité, et. al., 2009). A decoction of *Morinda morindoides* leaves is used in Democratic Republic of Congo traditional medicine against various diseases such as arthritis (Cimanga, 2003), malaria, amoebiasis, scabies, hemorrhoids, worms and gonorrhea (Mankele, et. al., 2006). In Ivory Coast, *Morinda morindoides* is used by the peoples of the west to treat diarrhoea of any kind (Bahi, 2000). For the demonstration of the antidiarrheal activity of the plant, several extraction solvents of different polarity are used. They focused groups of molecules more or less different. The purpose of this study is to identify and characterize phytomolecules relaxing smooth muscle of dichloromethane-ethanolic (two solvents of different polarity) extract of *Morinda morindoides*.

MATERIALS AND METHODS

Plant material

The leaves of *Morinda morindoides* were collected from Daloa (central west region of Ivory Coast) in April 2008. The plant was identified taxonomically and authenticated by Pr AKE ASSI, of the Department of Botany, University of Cocody, Abidjan, Ivory Coast. A voucher specimen (NO 17710) was deposited at the herbarium of the National Floristique center of the University of Cocody-Abidjan.

Preparation of plant materials

The leaves of *Morinda morindoides* were cleaned of extraneous matter, air-dried at room temperature for 7 days and ground into a fine powder. The dichloromethane-ethanolic extract of *Morinda morindoides* was prepared by the method described by Guede-Guina, et. al., (1993). To obtain the extract, the powder was dissolved in 800 ml of a mixture of dichloromethane / ethanol (200 ml dichloromethane and 600 ml of ethanol). The mixture was homogenized for 24 h at room temperature (25-30°C) using a magnetic stirrer IKAMAG-RCT. The homogenate obtained was filtered twice through cotton wool, then through Wathman filter paper (N°1). The filtrate was evaporated at 38°C to dryness using a rotary evaporator (BÜCHI). The resulting dry powder was taken as the dichloromethane-ethanolic extract of *Morinda morindoides* (ETDE).

Animals

Rabbit (crossbreed) of both sexes weighting between 1500 and 2000 g were used. All animals were kept in standard metal cage in standard environmental conditions and allowed to acclimatize for 2 weeks in our laboratory of Pharmacology and Biochemistry before experiments were started. They were provided with food and water ad libitum. All experiments were in compliance with the European Council legislation 87/609/EEC for the protection of experimental animals.

Drugs

Acetylcholine (Sigma chemical, USA), Propranolol (Astrazanecca Reims, France) were used.

Preliminary phytochemical analyses of the plant.

Freshly prepared extract was chemically tested qualitatively for the presence of chemical constituents. These were identified by characteristic colour changes using standard procedures (Trease and Evans, 1983).

Dose-response effect of the extract on the ileum contractile activity

The isolated tissue experiments were carried out as described (Gilani *et al.*, 1994). The animals had free access to water but were fasted for 24 h before experiment. The animals were sacrificed by a blow on the head, the abdomen was cut open and the ileum portion isolated out. Preparations of 3 cm length were mounted in 10 ml tissue baths containing Mac Ewen's solution of the following composition (mMol/L): [NaCl (130.05), KCl (5.63), CaCl₂ (2.16), PO₄H₂Na₂ (0.91), CO₃HNa (2.38), MgCl₂ (53) and glucose (11.11)] at a temperature of 37°C (±1°C), and aerated with air. The ileum was suspended in a 150 ml organ bath containing Mac Ewen's solution. A load of 0.5 g was applied and 1 h equilibration time was allowed during which the physiological solution was changed every 15 min. Changes in the tension were recorded with Ugo Basile microdynamometer with isotonic transducer (Amos *et al.*, 1998). At the end of equilibration period, the responses of the tissue to serial concentrations of acetylcholine (1.3×10^{-5} , 1.3×10^{-4} and 1.3×10^{-3} mg/ml), the extract (49×10^{-3} ; 190×10^{-3} ; 290×10^{-3} ; 390×10^{-3} mg/ml) were recorded. Furthermore, a fixed concentration of acetylcholine (1.3×10^{-3} mg/ml), and propranolol (1.3×10^{-5} mg/ml) were interacted with varying concentrations of the extract (49×10^{-3} ; 190×10^{-3} ; 290×10^{-3} ; 390×10^{-3} mg/ml). Each concentration tested was allowed a contact time of 1 min followed by washing three times. A resting period of 15 min was allowed before the next addition.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Dennett's t-test using Instat (Graph Pad 5.0 Software, U.S.A). At 95% confidence interval $P < 0.05$ was considered statistically significant.

RESULTS

Phytochemical Screening: The preliminary phytochemical screening of the extract revealed the presence of sterols and terpenes, polyphenols, flavonoids, tannins, saponins, and alkaloids but not confirm the presence of free or combined quinone compounds (Table 1).

Table 1: Phytochemical constituents of ETDE

Phytochemical constituents	Results
Sterols and terpenes	+
Polyphenols	+
Flavonoids	++
Tannins	++
Free or combined quinone compounds	-
Saponins	++
Alkaloids	+

+: phytochemical present -: phytochemical absent

Effect of ETDE on the isolated rabbit ileum

The effect of ETDE on the rabbit ileum were dose related. The extract at increasing concentrations (49×10^{-3} to 390×10^{-3} mg/ml) relaxed the spontaneous contractions of the rabbit ileum. This myorelaxation is indicated into a decrease in contraction amplitude and displacement of basal tone (Figure1). This effect of ETDE on the isolated ileum of rabbit was concentration-dependent with EC_{50} value (95% confidence limits) of $194 \pm 1,5 \times 10^{-3}$ mg/ml ($178,2 \times 10^{-3}$ - $212,2 \times 10^{-3}$, $n=3$) (Figure 2). There was 100% relaxation at concentration of 390×10^{-3} mg/ml.

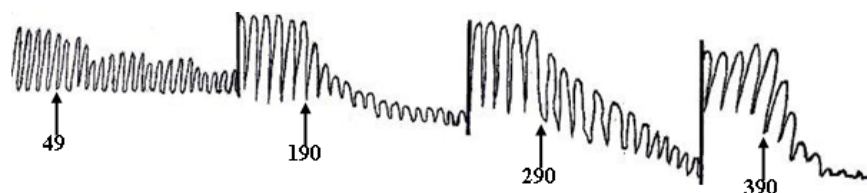


Figure 1: Effect of ETDE ($\times 10^{-3}$ mg/ml) on contractile responses in the isolated rabbit ileum
The extract produced a significant reduction in the amplitude of spontaneous contraction.

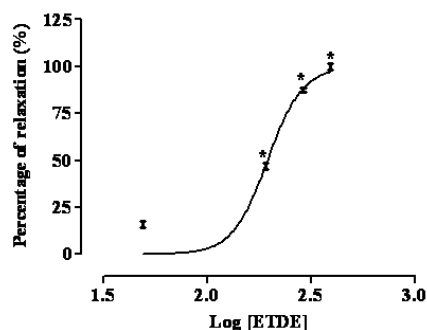


Figure 2: Dose-response curve for ETDE on the isolated rabbit ileum. The EC_{50} value was derived from this sigmoid dose-response curve. Values are expressed as percentage decrease of control (mean \pm SEM, $n = 3$, $*P < 0, 05$). $EC_{50} = 194 \times 10^{-3}$ mg/ml, IC 95% confidence intervals [$178,2 \times 10^{-3}$ - $212,2 \times 10^{-3}$].

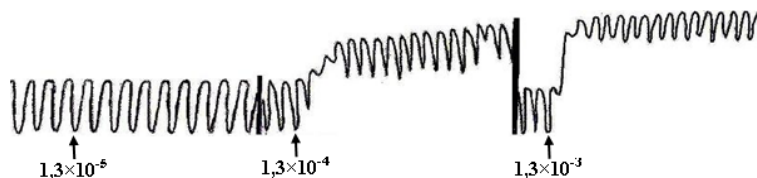


Figure 3: Effect of acetylcholine (mg/ml) on contractile responses in the isolated rabbit ileum. The ACh produced a significant increase in the amplitude of spontaneous contraction.

Effect of acetylcholine on the isolated rabbit ileum

Application of acetylcholine to the bathing medium of the isolated rabbit ileum, at increasing concentrations greatly increased the contractions of the tissue (Figure 3). This myostimulant action of acetylcholine was characterized by the elevations of the rate of contractions and the basal tone (Figure 3). When ACh was used at $1,3 \times 10^{-3}$ mg/ml the increase of the amplitude of the contractions was 100%. The calculated EC_{50} (95% confidence limits) was $235 \pm 1,13 \times 10^{-5}$ mg/ml ($123 \times 10^{-5} - 448 \times 10^{-5}$, $n=3$).

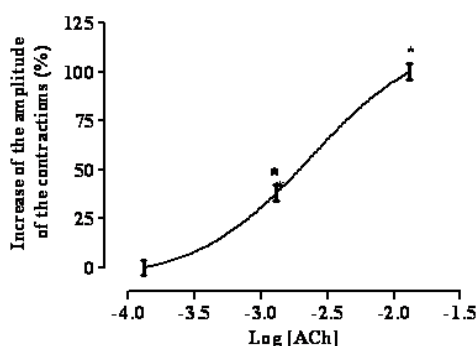


Figure 4: Dose-response curve for acetylcholine (mg/ml) on the isolated rabbit ileum. Values are expressed as percentage increase of control (mean \pm SEM, $n = 3$). $EC_{50} = 235 \times 10^{-5}$ mg/ml, IC 95% confidence intervals [$123 \times 10^{-5} - 448 \times 10^{-5}$].

Effect of interaction extract / acetylcholine on the isolated rabbit ileum

The extract concentration-dependently (190×10^{-3} ; 290×10^{-3} ; 390×10^{-3} mg/ml) reduced the effect of ACh ($1,3 \times 10^{-3}$ mg/ml), significantly (ANOVA, $P < 0.05$). This inhibitory effect of ETDE showed the reduction frequency and amplitude of spontaneous contractions from 29,42% at 190×10^{-3} mg/ml up to maximal response 96.32% at 390×10^{-3} mg/ml (Figure 5).

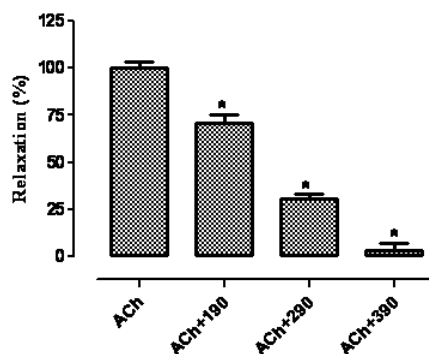


Figure 5: Effect of ETDE ($\times 10^{-3}$ mg/ml) on contractile responses in the isolated rabbit ileum induced by ACh (mg/ml). Values are expressed as percentage decrease of control (mean \pm SEM, $n = 3$, $*P < 0,05$).

Effect of interaction propranolol/extract on the isolated rabbit ileum

Incubation of ileum with propranolol ($1,3 \times 10^{-2}$ mg/ml) as a adrenergic receptor antagonist did not alter the inhibitory effect of the ETDE. Interact with propranolol, ETDE induced the decrease of the amplitudes of contraction (Figure 6). Increasing concentrations of the extract (190×10^{-3} ; 290×10^{-3} ; 390×10^{-3} mg/ml) in the presence of propranolol caused significantly ($P < 0,05$) a decrease of the amplitude of contraction respectively from 37%, 75,5% to 97,8% (Figure 6).

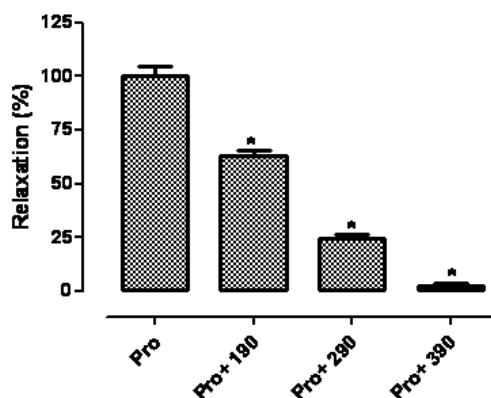


Figure 6: Antagonistic effect and dose-response of ETDE ($\times 10^{-3}$ mg/ml) on *in vitro* stimulation of ileum contractions initiated by propranolol. Values are expressed as percentage decrease of control (mean \pm SEM, n = 3, * $P < 0,05$).

DISCUSSION

The aim of the present study was to identify and characterize phytomolecules relaxing smooth muscle of dichloromethane-ethanolic extract of *Morinda morindoides*. Infection of the digestive tract, diarrhoea is evoked by hyperpropulsive motility of gastrointestinal tract and hypersecretion throughout the intestinal mucosa (Yegnanarayan and Shrotri, 1982). The acceleration of the contractility of the ileum is one aspect observed. ETDE concentration-dependently inhibited the spontaneous concentrations of the isolated rabbit ileum. This action is expressed with EC_{50} of $193 \pm 1,5 \times 10^{-3}$ dichloromethane-ethanolic extract of *Morinda morindoides* leaves induced a graded relaxation of the smooth muscle of the gastrointestinal tract, the severity of which depended on the concentration of the extract. This inhibition of spontaneous ileum contractions was identical to that of several plants with antidiarrhoeal properties such as *Mallotus oppositifolium* (Euphorbiaceae) (Kamgang, et. al., 2001), *Securinega virosa* (Euphorbiaceae) (Magali, et. al., 2007) etc. According to Brunton, (1996), the property of reducing intestinal contractions is demonstrated by most antidiarrhoeal agents, and helps in preventing excessive loss of fluid and ingesta. Early studies have also reported that antidiarrhoeal activity of medicinal plants may be due to alkaloids, saponins, tannins sterols and reducing sugar (Galvez, et. al., 1991; Longanga, et. al., 2000). Flavonoids and sugars obtained from selected traditional medicinal plants from some parts of the world, were reported by Rahman and Wilcock, (1991) and Palombo, (2005) respectively, and were shown to exhibit antidiarrhoeal properties. The antidiarrhoeal activity of flavonoids and tannins have been shown by Lutterodt, (1989) and Pousset, (1989). Indeed, the quercetol subdued in flavonoids reduced the contractions of intestine (Pousset, 1989; Tripathi, 1994). Some of these chemical groups such as flavonoids, saponins, alkaloids and tannins have been identified in the total aqueous extract, the ethanolic extract of *Morinda morindoides* (Bagré, 2004). The preliminary photochemical tests of the extract also carried out the leaf of *Morinda morindoides* revealed the presence of sterols and terpenes, polyphenols, flavonoids, tannins, free or combined quinone compounds, saponins, and alkaloids. Therefore, the antidiarrhoeal activity of the extract is most likely due to the presence of these chemical groups, which would act singly or in combination with other constituents present. Cimanga, (2010) has shown that the antispasmodic activity of the plant is due to the presence of quercetin, quercitrin epoxygaertneroside and gaertneroside.

To examine the possible mechanism of action of this active phytochemical constituents, the extract was used in interaction with the acetylcholine and the propanolol. substance contracture potential sensitivity often causes diarrhoea (Schmitt, 1978; Devor, et. al., 1992), on isolated rabbit ileum was attenuated or abolished by the extract in a concentration dependent fashion. There are a variety of cholinergic receptors: muscarinic and nicotinic receptors. Antagonists of these receptors also called atropinergic, exert antispasmodic action on smooth muscle. The antispasmodic action is manifested by decreased muscle tone, decreased the amplitude and velocity of peristaltic contractions. These effects are similar to those observed with the extract; the compounds of the extract would atropinergic. Bahi, (2000) showed that the relaxant activity of the total aqueous extract of *Morinda morindoides* was linked to simulation of catalytic activity of AChE that hydrolyses much faster ACh which can no longer reach the postsynaptic membrane. Otherwise according to Horowitz, et. al., (1996) it is likely that the extract was acting as an antagonist of the neurotransmitter to block its effect by preventing the release of Ca^{2+} from the cisternae and hence its entry into the cell to active smooth muscle contraction. Smooth muscle has several receptors other than cholinergic receptors. These could be used by some constituents to induce the relaxation. It was here that propranolol, a adrenergic receptor antagonist was used. The inhibitory effect of the extract on contractile responses in the isolated rabbit ileum was not modified by adrenergic receptor antagonist. Propanol concentration-dependently reduced amplitude of spontaneous concentrations of the isolated rabbit ileum, but did not displace the basic tone. This inhibition and reduced amplitudes of contractions and the displacement of basal tone recorded might assume that the compounds concentrated in the solvents used are not adrenomimetic. It would potentiate the relaxant effects of the extract. However the exact mechanism involved is difficult to clarify as control mechanisms of intestinal functions are various and imply intrinsic and extrinsic nerves and hormones (Bennett, 1992; Binder, 1992). The ability of most of the plant extract to attenuate the spontaneous contraction and its antimicrobial activity support the traditional use of the plant in controlling diarrhea.

CONCLUSION

In conclusion, the relaxant effect of the extract may be attributed to the phytochemical constituents such as sterols and terpenes, polyphenols, flavonoids, tannins, saponins, and alkaloids. These compounds would be atropinergic and could explain the antidiarrhoeal activity of the plant. However, more detailed phytochemical analysis are required to isolate and characterize each active compounds which are responsible for the antidiarrhoeal activity and exact mechanisms of action of this activity.

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Abbreviation

Dichloromethane-ethanolic extract of *Morinda morindoides*: ETDE

EC₅₀: efficient concentration 50%

Pro: propranolol

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