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Antispasmodic phytomedicine, from traditional utilization to rational formulation: functional approach

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Abstract

The aim of this study was to assess traditional preparation used to treat infantile colic and to propose a rational formulation. We studied the antispasmodic effect *in vitro* on rat intestine using isometric transducer and rationalized the traditional preparation using functional approach. The crude aqueous extract of fifteen plants induced dose-dependent relaxation in rat intestine with an IC_{50} of 164.7 ± 1.2 µg/mL. With a new rational formulation, the IC_{50} was 27.8 ± 1.1 µg/mL, a very effective value when compared to antispasmodic drugs e.g. loperamide (IC_{50} = 49.6 ± 1.1 µg/mL), trimebutine (IC_{50} = 106.5 ± 1.0 µg/mL), phloroglucinol (IC_{50} = 119.1 ± 1.1 µg/mL), and pinaverium bromide (IC_{50} = 837.7 ± 1.1 µg/mL). This study validates traditional utilization the mixture as antispasmodic and demonstrates that functional approach can be used to optimize traditional preparation.

Keywords: Antispasmodic, Rat small intestine, infantile colic, medicinal plants, traditional medicine, phytotherapy.

Introduction

It is well know that excessive infant crying can be accompanied by abdominal pain as the result of gas, cramping, spasms and abnormal intestine contraction (Lobo et al., 2004). It is one of the most frequent visits to the paediatric emergency of new born babies (Calado et

al., 2009). Traditional practitioners in Morocco usually use the traditional infusion of fifteen plants against infantile excessive crying. Despite its benign, natural course, inconsolable crying is a significant problem in infants and imparts a psychological, emotional, and physical burden to parents.

Many different treatments have been suggested including pharmacotherapy, dietary therapy and sensory stimulation. We focus attention on phytomedicine because it is the treatment widely used in Africa. Some herbal teas act as antispasmodics (Forster et al., 1980). In a double-blind study, herbal tea containing extracts from several herbs, including chamomile (McKay and Blumberg, 2006), vervain (Pascual et al., 2001), licorice, fennel (Alexandrovich et al., 2003; Savino et al., 2008), and balm mint, improved colic and abdominal pain significantly more often than a placebo (Weizman et al., 1993). Fennel seed oil has been shown to reduce intestinal spasms and increase motility of the small intestine (Capasso et al., 2007; Savino et al., 2008). Recently, the use of fennel tea has been discouraged because of its mutagenic effect on bacteria and carcinogenic effect on mice (Iten and Saller, 2004). The aim of this study was to evaluate the antispasmodic effect of each plant which composed this preparation and to propose a rational formulation by functional approach.

Material and Methods

Chemicals

All reagents were purchased and used for research use only. Carbamylcholine hydrochloride, naloxone (Sigma-Aldrich, St-Quentin Fallavier, France), phloroglucinol (Cephalon, Maisons-Alfort, France), trimebutine and loperamide (TAVA, Paris la défense, France), pinaverium bromide (Solvay Pharma, Suresnes, France).

Plant resources and preparation of crude extract

The plant infusion was prepared according to the method recommended traditionally for oral administration to treat infantile colic. All the plants were purchased from a local herb store and were authenticated at the Scientific Institute of Rabat – in Morocco. A voucher specimen (table 1) was deposited in the herbarium of the Institute except Illicium verum which was purchased from Cepasco (13420 Gémenos, France). Crude extract was obtained by the maceration of dried plant in boiling distilled water (1g/10ml), and store at room temperature for 24 hours. The aqueous extract was filtered, concentrated to obtain final concentration (w/w) as shown in Table 1, and kept at 4°C until use.

Animals

Wistar rats weighing 177–200g (Janvier, Le Genest-St-Isle, France) were kept in cages under standard laboratory conditions with tap water and standard rat chow ad libitum, in a 12-h/12-h light/dark cycle at a temperature of 21–23°C. The study was conducted in accordance with accepted principles outlined in the “Guide for the Care and Use of Laboratory Animals” prepared by the National Academy of Sciences and published by the National Institutes of Health and efforts were made to minimize animal suffering and number

Table 1. List of plants used in traditional medicine.

	Name	Family	Part of plant used	Voucher numbers	Final concentration W/W (%)
1	Zygophyllum gaetulum Emb.	Zygophyllaceae	Leaves, stem	RAB77469	8.44
2	Cuminum cyminum L.	Apiaceae	seeds	RAB39598	7.29
3	Ammodocus leucotrichus L.	Apiaceae	seeds	RAB61594	3.16
4	Carum carvi L.	Apiaceae	seeds	RAB39463	6.27
5	Rosarinus officinalis L.	Lamiaceae	Leaves, flowers	RAB62750	1.21
6	Illicium verum	Magnoliaceae	Fruit, seeds	French store	6.83
7	Artemisia herba alba Asso.	Asteraceae	foliage	RAB76713	2.37
8	Foeniculum vulgare Mill.	Apiaceae	seeds	RAB39316	4.26
9	Mentha pulegium L.	Lamiaceae	Leaves, stem	RAB34027	3.97
10	Pimpinella anisum L.	Apiaceae	seeds	RAB38931	5.45
11	Lavandula angustifolia Chaix.	Lamiaceae	Leaves, flowers	RAB33858	6.85
12	Lippia citriodora Lam.	Verbenaceae	foliage	RAB45756	4.57
13	Punica granatum L.	Punicaceae	Pericarp	RAB65559	8.18
14	Nigella sativa L.	Renonculaceae	seeds	RAB10359	5.76
15	Origanum vulgare L.	Lamiaceae	leaves, stem	RAB7158	3.94

The plant samples were purchased in Morocco local herb store. Only *Illicium verum* was purchased in French local store.

of animals used. Ethics approval was obtained from Université Paris Diderot- Paris 7.

Preparation of isolated rat jejunum

Male adult rats were fasted 16 h with water ad libitum. Animals were killed by vertebral dislocation. The proximal jejunum (5 cm distal from the ligament of Treitz) was dissected out and rinsed in cold saline solution. The mesenteric border was carefully stripped off using forceps. Segments of the jejunum contents, about 2.0 cm long were removed by flushing with Tyrode's solution of the following composition in millimoles (mM): NaCl (136.9), KCl (2.7), $CaCl_2$ (1.8), $NaHCO_3$ (11.09), $MgCl_2$ (1.05), NaH_2PO_4 (0.42), glucose (5.5) at pH 7.4. The tissue was mounted in a 7 mL organ bath containing Tyrode's solution maintained at 37°C ± 0.5 and gassed with 95% O_2 and 5% CO_2. An initial tension of 1g was applied and the spontaneous muscular contractility was simultaneously recorded isometrically using Ugo Basile Unirecorder 7050 (Cortes et al., 2006). Drugs and extract were added directly to the organ chamber in volumes not exceeding 1% of the total bath volume. At the end of the 45-min equilibration period, the effect of different drugs and/or crude extracts of mixture or different plants were investigated cumulatively with a contact time for each concentration of 2 min.

Determination of functional ratio

The functional approach to build a rational formulation consists in determining the concentration of extract for each plant giving half maximal inhibitory response (IC_{50}). After this determination, only the plants with the half maximal response lower than the mixture of plants were used to build rational formulation. The rational formulation was obtained on the basis of functional ratio (F_r), which is considered as a unit of preparation of a new rational formulation. The F_r was obtained using the following equation:

$$F_r = [{}_xIC_{50} / {}_yIC_{50}]$$

${}_xIC_{50}$ represent the value of IC_{50} of plant x, and ${}_yIC_{50}$, the lowest IC_{50} found for the most effective plant extract (y) among plants which composed traditional preparation.

Statistical analysis

All the data are expressed as mean ± standard error of mean (S.E.M., *n* = number of experiments), except for the IC_{50} (concentration of drugs causing half-maximal responses), which are presented as geometric means accompanied by their respective 95% confidence intervals. The statistical analyses were obtained by the one way analysis of variance (ANOVA), followed by the Dunnett's test where necessary. $P<0.05$ was considered significant. The concentration–response curves were analyzed by non-linear regression (Graphpad program for Windows version 5.01. Graphpad, San Diego, CA, USA).

Results

Screening of plants

The first step of the study was to examine the intrinsic activity of the mixture (M15)

and each plant which composes traditional preparation. For each extract we measured the amplitude of spontaneous contraction, frequency of spontaneous contraction, relaxation and contraction of tissue before and after addition of the plant extract cumulatively. Typical recording of the effect of M15 on jejunum is presented in Figure 1. In control condition, the frequency of spontaneous contraction of jejunum was close to 32 contractions per minute. The amplitude of spontaneous contraction of tissue (A) without any addition of product was evaluated. This amplitude was stable (B) before addition of 50 µg/mL of M15, then the amplitude decreased with the augmentation of concentration (C, D). With concentrations of up to 100 µg/mL, the frequency of contraction declined and became significantly blunted with 1550 µg/mL of M15 compared to that obtained with 500 µg/mL of M6. Further addition of 10^{-6} M of Carbamycholine (Carb) induced contraction of the tissue pre-treated with M15 and M6.

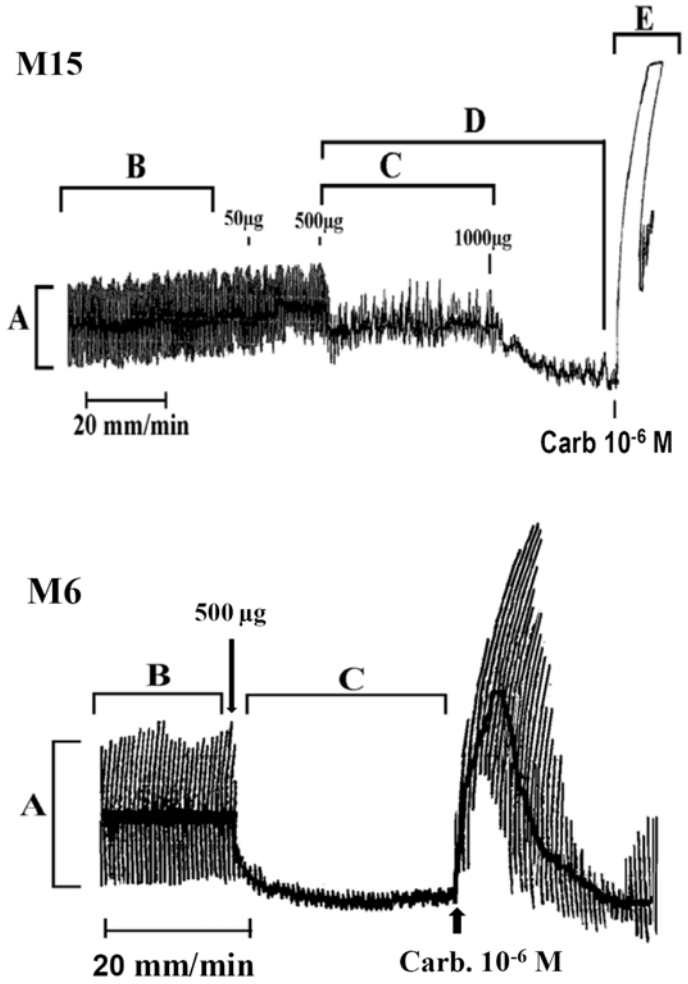

Figure 1: The Typical recording of the effect of traditional preparation and rational formulation on rat jejunum. Amplitude of spontaneous contraction is indicated by A, and B wave of the spontaneous contraction of intestine. C. Decrease of contraction's frequencies after addition of 500 µg/mL of M15. Relaxation of intestine is represented by D whereas E was the contraction induced by 10^{-6} M of Carbamylcholine (Carb). M6, addition of Carbamylcholine provoked transient contractions of tissues without suppression of wave of spontaneous contractions.

Table 2. Functional ratio

	Name	Abbrev	IC_{50} (µg/mL)	Functional ratio (F_r)
1	Cuminum cyminum L.	Cum	484.1 ± 1.0	6.89
2	Artemisia berba alba	Art	137.0 ± 1.1	1.95
3	Lavandula angustifolia	Lav	111.0 ± 1.2	1.58
4	Mentha pulegium L	Men	149.7 ± 1.1	2.13
5	Origanum vulgare L	Orig	204.3 ± 1.0	2.91
6	Rosarimus officinalis L	Ros	93.7 ± 1.2	1.33
7	Illicium verum	illv	603.3 ± 1.0	8.59
8	Punica granatum L	Pun	201.5 ± 1.2	2.87
9	Nigella sativa L	Nig	737.1 ± 1.1	10.49
10	Lippia citriodora Lam	Lip	70.2 ± 1.1	1
11	Zygophyllum gaetulum Emb	Zyg	103.5 ± 1.2	1.47
12	Artemisia, Lavandula, Mentha, Rosmarinus, Lippia, Zygophyllum, Origanum, illicium, Punica, Nigella, Ammodancus, Carum, Foeniculum, Pimpinella, Cuminum	M15	164.7 ± 1.2	2.34
13	Artemisia, Lavandula, Mentha, Rosmarinus, Lippia, Zygophyllum	M6	27.5 ± 1.1	0.39
14	Cuminum cyminum L.	Cum	484.1 ± 1.0	6.89
15	Artemisia berba alba	Art	137.0 ± 1.1	1.95

Values of IC_{50} are expressed as geometric mean with 95 % confidence intervals

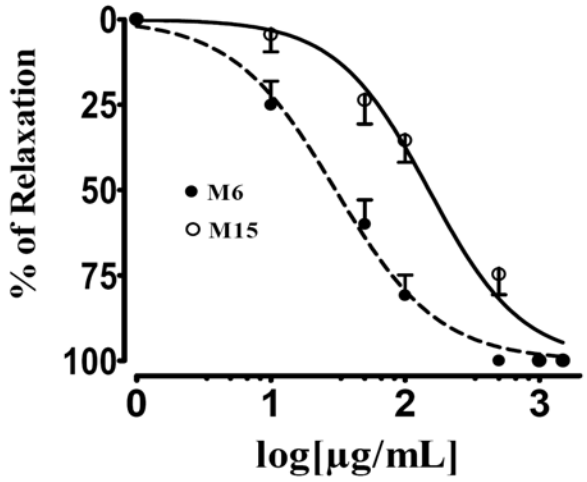

Figure 2: Comparison of the effect of traditional preparation and rational formula. The effect of M6 is more effective in relaxation of intestine (IC_{50}=27.5 ± 1.1 µg/mL) than M15 (IC_{50} = 164.7 ± 1.2 µg/mL). Values of IC_{50} are expressed as geometric mean with 95 % confidence intervals. n= 7 tissues studied.

Studies of antispasmodic plants

The traditional preparation studied contains eleven plants which clearly provoked significant antispasmodic effect on rat intestine, namely *Cuminum cyminum, Artemisia berba alba, Lavandula angustifolia, Mentha pulegium, Origanum vulgare, Rosarimus officinalis, Illicium verum, Punica granatum, Nigella sativa, Lippia citriodora, Zygophyllum gaetulum.* Table 2 shows that the effect of *Lippia citriodora* was more important (IC_{50} = 70.2 ± 1.1 µg/mL) than that of *Rosarinus officinalis* (IC_{50} = 93.7 ± 1.2 µg/mL) and *Zygophyllum gaetulum* (IC_{50} = 103.5 ± 1.2 µg/mL), whereas *Nigella sativa, Illicium verum* and *Cuminum cyminum*, exhibited weak effects (respectively IC_{50}: 737.1 ± 1.2, 603.3 ± 1.0 and 484.1 ± 1.0 µg/mL).

Functional approach formulation

The functional approach formulation was used in this study in order to obtain rational new formulation (M6 or Liberaline®) of traditional remedy (M15). The functional approach consists in choosing among the different plants, those that induced intestine relaxation with an IC_{50} lower than that of traditional preparation (M15). According to these criteria, only six plants were selected (table 2). The lowest IC_{50} was found with *Lippia citriodora* (IC_{50} of 70.2 ± 1.1 µg/mL) and the value of 1 was given to this extract whereas the highest functional ratio of 2.13 was attributed to *Mentha pulegium.* Following this method we obtained a new formula namely M6 containing *Artemisia berba alba, Lavandula angustifolia, Mentha pulegium, Rosmarinus officinalis, Lippia citriodora, Zygophyllum gaetulum.* This new formulation was found to be more effective in intestine relaxation (IC_{50}=27.5 ± 1.1 µg/mL) than the mixture M15 (IC_{50} = 164.7 ± 1.2 µg/mL) as shown the figure 2. We compared the effect of M6 on amplitude and frequency on spontaneous contraction of the intestine (Fig.3). The figure 4A and 4B shows the different results obtained. We found that M6 affected only

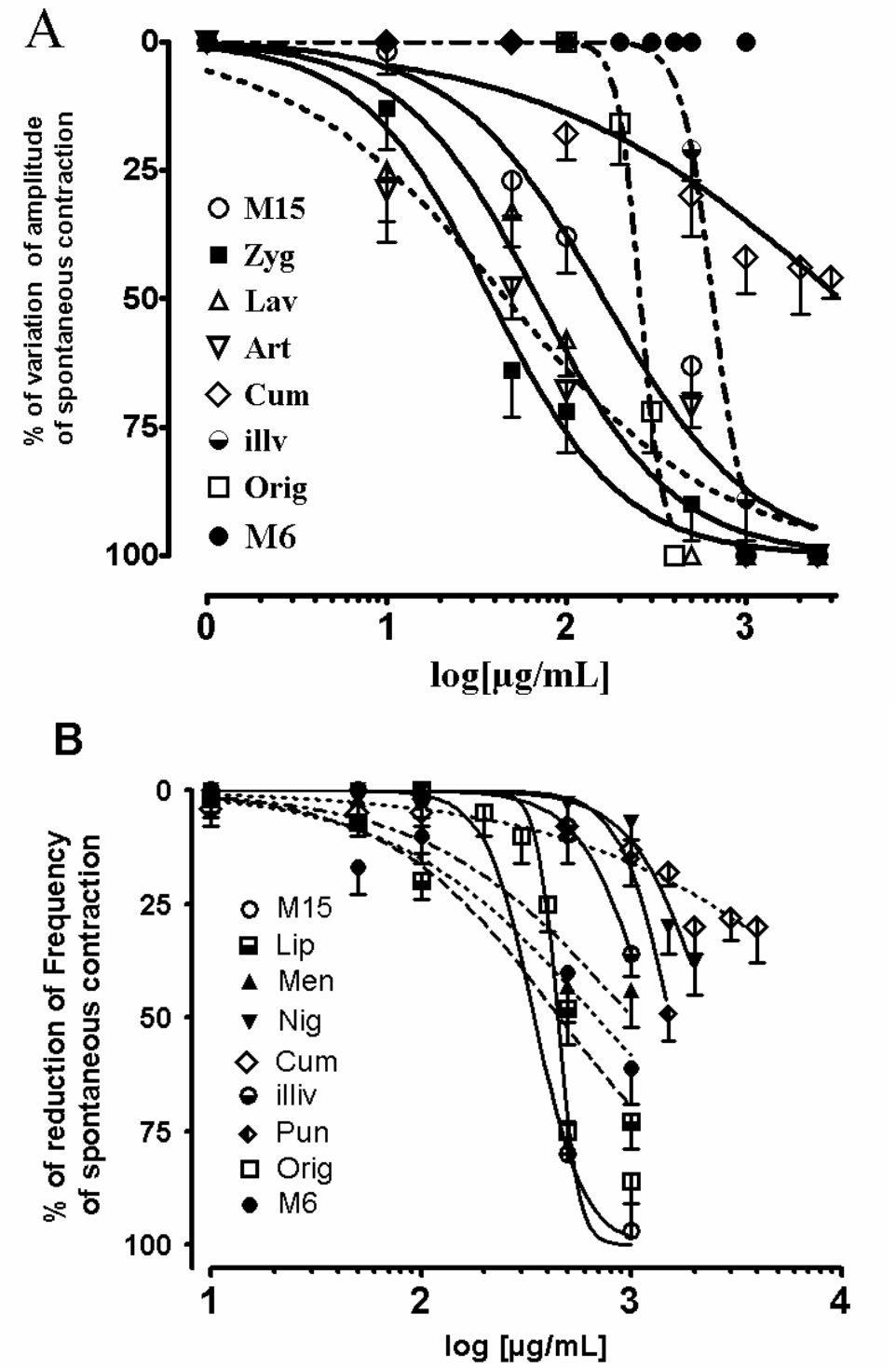

Figure 3: Comparison of the effect of rational formulation with others extracts of plants. Effect of M6 on amplitude (A) and frequency (B) on intestinal contractions, when compared with others extracts of plants. The total suppression of the effect at maximal concentration of extract was considered as 100% of inhibition. Values expressed as geometric mean with 95 % confidence intervals. n= 7 tissues studied.

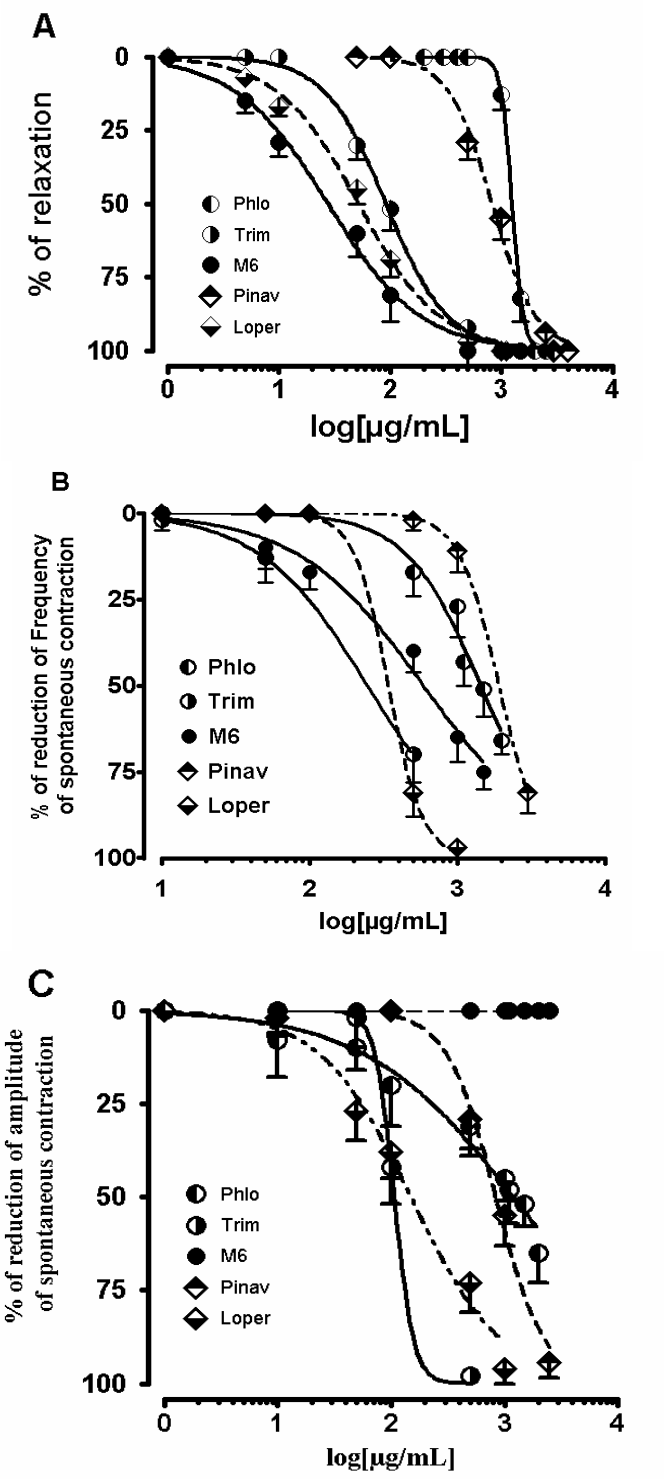

Figure 4: Comparison of rational formulation with medicine used in clinic. The effect of different extracts of plants on relaxation (panel A), frequency (panel B) and amplitude (panel C) of rat intestine. Phlo: phloroglucinol, Trim: trimebutine, Pinav: pinaverium bromide, Loper: loperamide, M6. The maximun effect obtained with each drug was considered as 100%. Values expressed as geometric mean with 95 % confidence intervals. n= 6 tissues studied.

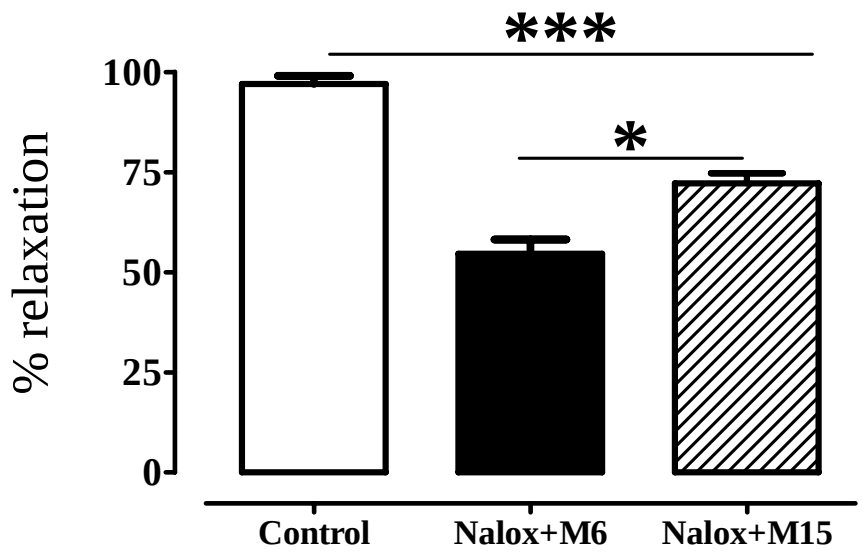

Figure 5: Effect of naloxone on rational formulation. Intestine relaxation induced by 500 µg/mL of M6 and 1500 µg/mL of M15 after the pre-treatment of the tissue with 10^{-4} M of naloxone (Nalox). The control was obtained with the same concentration of M6 and M15 without pre-treatment with naloxone and was considered as 100% effect. The statistical analyses were obtained by the one way analysis of variance (ANOVA), followed by the Dunnett's test. *P<0.01, ***p<0.001, n = 6 tissues studied.

the frequency of the spontaneous contraction whereas M15 induced modification both in frequency and amplitude.

Comparison of new formulation M6 with antispasmodics drugs

The effect of new formula M6 was compared to some drugs usually used in clinics such as loperamide (Imodium®), phloroglucinol (Spasfon®) trimebutine (Débridat®) and pinaverium bromide (Dicetel®), according to their effect on frequency and amplitude of spontaneous contraction and on intestinal relaxation. The result showed that M6 was more effective in the relaxation of rat small intestine than loperamide, phloroglucinol, trimebutine and pinaverium bromide (Fig.4). The effect of M6 was more important than that of phloroglucinol and pinaverium bromide in term of frequency and contraction (Fig. 4A, 4B), and without effect in amplitude of contraction (Fig. 4C). Table 3 summarizes the different values of IC_{50} on relaxation, frequency and amplitude of spontaneous contractions. When the tissue was pre-treated by 10^{-4} M naloxone, the biological effect of M6 and M15 was markedly reduced 54.60 ± 7.98 and 72.20 ± 5.58 respectively p<0.001, compared to control (Fig. 5), suggesting that this effect on rat intestine may partially involved opioid receptors.

Discussions

The present study demonstrates an antispasmodic effect of some components of traditional preparation. This study shows that a new preparation obtained from a rationale

Table 3. Comparison of M6 with antispasmodic medicines

Products		**Relaxation**	**Frequency**	**Amplitude**
Name	Commercial name	IC_{50} (µg/mL)	IC_{50} (µg/mL)	IC_{50} (µg/mL)
Phloroglucinol	Spasfon®	1252.0 ± 0.2	1440.0 ± 0.5	1197.1 ± 1.1
Trimebutine	Débridat®	91.8 ± 0.1	246.0 ± 0.1	106.5 ± 1.2
Pinaverium bromide	Dicetel®	826.8 ± 0.3	1910.0 ± 0.1	837.7 ± 1.1
Loperamide	Diaretyl®	51.8 ± 0.1	341.4 ± 0.8	145.8 ± 1.1
Artemisia, Lavandula, Mentha, Rosmarinus, Lippia, Zygophyllum	M6	27.5 ± 1.1	587.7 ± 0.1	-

Values of IC_{50} are expressed as geometric mean with 95 % confidence intervals

reevaluation of physiological effect can be more effective than the original. The crude extract of fifteen plants (M15) was obtained by infusion of the plant in the same manner of the preparation of the tea. This form of the preparation is the commonly used herbal medicine to treat gastrointestinal disorders. The results of the present study clearly demonstrate that *in vitro* pre-treatment of rat intestine with the aqueous crude extract of M15 induced dose-dependent relaxation. In addition, this relaxation was accompanied by a reduction of frequency and amplitude of spontaneous contractions. Further addition of Carbamylcholine after maximal effect of M15 showed that this effect was reversible. After confirmation of traditional utilisation of M15, we focused our study on the role of each plant in multi-component mixture extracts of M15. Among the fifteen plants of M15, we found that only eleven plants can provoke relaxation of rat intestine (Table 3).

Most of those antispasmodics plants such as *Lippia citriodora* or vervein (Forster et al., 1980; Pascual et al., 2001), *Rosmarimus officinalis* (Al-Sereiti et al., 1999), *Mentha* (Lopez et al., 2009; Spirling and Daniels, 2001), *Origanum vulgare* (Arcila-Lozano et al., 2004) were already used alone to treat infantile colic or gastrointestinal disorders. In traditional preparation, we also found a plant previously demonstrated as spasmodic when used alone or in mixture of multicomponent, such as *Foeniculum vulgare* (Alexandrovich et al., 2003; Savino et al., 2008). The result we obtained in this study with *Pimpinella anisum* in rat small intestine was contrasting with common consideration that this plant exhibits antispasmodic and relaxant effect on smooth muscle. Several studies previously showed the relaxant effect of an antispasmodic effect of aqueous and ethanol extract of *Pimpinella anisum* on isolated guinea pig tracheal chains (Boskabady and Ramazani-Assari, 2001). A similar effect was found on rat ococcygeus smooth muscle with hydroalcoolic extract (Tirapelli et al., 2007). In our study we clearly showed that aqueous extract of *Pimpinella anisum* significantly increases amplitude and frequency of spontaneous contractions of rat small intestine. Same data were obtained with higher concentration of *Carum carvi* (up to 3 mg/mL). It is noteworthy that several spasmodic plants in traditional medicine including *Carum carvi, Pimpinella anisum* and *Foeniculum vulgare* share common constituents i.e. quercetin 3-glucuronide, isoquercitrin, quercetin 3-O-caffeylglucoside, quercetin 3-

arabinoside. According to Kunzemann (Kunzemann and Herrmann, 1977), the following compounds were obtained from the former plants Quercetin 3-glucuronide from caraway (*Carum carvi L.)*, fennel (*Foeniculum vulgare Mill*.), anise (*Pimpinella anisum L*.); isoquercitrin from caraway and fennel; rutin from fennel and anise; quercetin 3-O-caffeylglucoside and kaempferol 3-glucoside from caraway; quercetin 3-arabinoside from fennel, and luteolin 7-glucoside, isoorientin and isovitexin from anise. Other constituents were identified by the usual procedures such as kaempferol 3-glucuronide and kaempferol 3-arabinoside in fennel, apigenin 7-glucoside and a luteolin glycoside in anise. Glycosides contained in the fruit of the three spices are also present in the leaves. Leaves of caraway and fennel in addition contain isorhammetin glycosides in low concentration. Aqueous extract of radish seeds which tested positive for terpenes, flavonoids, phenols, alkaloids and saponins, showed a spasmogenic effect in isolated rabbit jejunum and ileum (Ghayur et al., 2005). Several studies *in vitro* and *in vivo* showed that those compounds can regulate disturbances of gastrointestinal tract such as inhibition of guinea pig intestinal peristalsis by the flavonoids quercetin, naringenin, apigenin and genistein (Gharzouli and Holzer, 2004). Their effects depend on the mechanism involved and the tissue. The flavonoid vitexin was considered as spasmolytic in rat duodenum whereas isovitexin was devoid of activity (Ragone et al., 2007). Flavonoid from licorice isoliquiritigenin plays a dual role in regulating gastrointestinal motility, both spasmogenic and spasmolytic. The spasmogenic effect may involve the activating of muscarinic receptors, while the spasmolytic effect is predominantly due to blockade of the calcium channels (Chen et al., 2009). Saponins were found to possess antispasmodic activity in the guinea pig isolated ileum (Corea et al., 2005). Quercetin, quercitrin and rutin from *Morinda morindoides* leaves exhibited antispasmodic effect, whereas the saponins of the same plant displayed spasmogenic effect on guinea-pig isolated ileum (Cimanga et al., 2010). The benefit effects of infantile colic of antispasmodic and relaxant plants extracts may be justified by their analgesic and anti-inflammatory properties. Several studies confirmed anti-inflammatory and analgesic properties of *Lavandula angustifolia Mill* (Ching, 1999; Hajhashemi et al., 2003), *Lippia citriodora* Lam (Calvo, 2006; Speroni et al., 2007), *Zygophyllum gaetulum* (Rimbau et al., 1999) and *Nigella sativa* (Al-Naggar et al., 2003).

Another aim of this study was to proposed rational formulation of traditional medicine using a functional approach. This was used to rationalize formulation of traditional medicine during the process of development of phytomedicine according to the recommendation of world health organisation (WHO) and African intellectual property organization (AIPO) in 2004 (WHO/AIPO, 2004). The method consists in determining for each plant, the concentration of extract that gives half-maximal response (IC_{50}). After this determination, only the plants exhibiting an IC_{50} lower than traditional formulas were used to build a rational formulation. The rational formulation of herbal medicine is obtained on the basis of functional ratio, which is the ratio of IC_{50} as showed in equation. This method usually attributes the plant extract that gives the lowest IC_{50} a functional ratio of 1. In this study, only six plants were retained to build rational formulation according to their IC_{50} (lower than traditional medicine see table 2). This method allowed us to obtain new herbal medicine formulation with IC_{50} of 27.5±1.1 µg/mL. This IC_{50} was 6 fold more potent than traditional preparation'one (IC_{50} = 164.7 ± 1.2 µg/mL). M6 induced relaxation of intestine and reduced frequency of spontaneous contraction without affecting amplitude. Here we confirm that a simple pharmacological approach is useful to identify new potential drugs

from traditional remedies.

The present antispasmodic effect compare favorably with commercially available products including trimebutine, loperamide, phloroglucinol and pinaverium bromide. The result showed that, in the rat model, although the effect of M6 was weak compared to that of trimebutine and loperamide in term of frequency, (see Table 3), M6 was more effective to induce relaxation than current remedies used in clinics.

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Conclusion

In conclusion our study provides novel evidence in support of the traditional use of traditional aqueous extract of M15 in infantile colic remedy as a symptom of intestinal contraction and pain. In addition this study clearly demonstrates that functional approach can be used to ameliorate traditional preparation to rational formulation during the development of phytomedicine according to the recommendation of AIPO (AIPO, 2002).

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