Revista Brasileira de Farmacognosia
Brazilian Journal of Pharmacognosy
18(1): 16-20, Jan./Mar. 2008
Received 30 August 2007; Accepted 29 January 2008

Artigo

Choleretic and antispasmodic effects of *Lippia integrifolia* aqueous extract

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RESUMO: "Efeitos colerético e antiespasmódico do extrato aquoso de *Lippia integrifolia*". O extrato aquoso das partes aéreas de *Lippia integrifolia* foi ensaiado quanto aos seus efeitos colerético e antiespasmódico. Doses de 250, 500 e 750 mg/kg administradas oralmente em ratos aumentaram significativamente o fluxo biliar e a saída de ácidos biliares. O extrato também exibiu uma significativa redução das contrações induzidas por acetilcolina, $CaCl_2$ e KCl em jejuno isolado de rato. O conteúdo total de derivados dos ácidos cafeoilquínicos, expressado como ácido clorogênico foi de 0.10% w/v através de determinação espectrofotométrica.

Unitermos: *Lippia integrifolia*, Verbenaceae, efeito colerético, efeito antiespasmódico.

ABSTRACT: The aqueous extract of the aerial parts of *Lippia integrifolia* has been assayed for its choleretic and antispasmodic effects. Doses of 250, 500 and 750 mg/kg administered orally in rats significantly increased the bile flow and the bile acid output. The extract also showed a significant reduction of the contractions induced by acetylcholine, $CaCl_2$ and KCl on isolated rat jejunum. The total caffeoyl quinic acids derivatives content, expressed as chlorogenic acid was 0.10% w/v by spectrophotometric determination.

Keywords: *Lippia integrifolia*, Verbenaceae, choleretic effect, antispasmodic effect.

INTRODUCTION

The genus *Lippia*, from the Verbenaceae family, includes approximately 200 species of herbs, shrubs and small trees mainly distributed in South and Central America and tropical Africa. Traditional uses of 52 medicinal species, belonging to this genus, have been extensively revised (Pascual et al., 2001). Most of them are mainly employed for the treatment of respiratory and gastrointestinal disorders (Agra et al., 2007). Despite this, there are scarce pharmacological reports which could provide scientific validation of their popular uses (Silva et al., 2006; Oliveira et al., 2006; Sena-Filho et al., 2006).

Six of about 31 species of *Lippia* growing in North and Central Argentina (Zuloaga, 1999), are indicated for gastrointestinal disorders as decoctions: *Lippia alba*, *L. asperifolia, L. fissicalyx*, *L. grisebachiana, L. integrifolia* and *L. turbinata* (Rondina et al., 2003).

Among them, *Lippia integrifolia* (Gris.) Hieronymus (Verbenaceae) is an aromatic shrub, known popularly as "pulco", "poleo", "incayuyo", "té del inca", "manzanilla", "manzanillo" and "inca yerba", that grows in La Rioja, San Juan, Catamarca, Salta, Jujuy, Tucumán and Córdoba Provinces. The decoctions of leaves and flowers are traditionally used against dyspepsia, indigestions and stomachaches, as diuretic, emmenagogue, antibiotic (for gonorrhoeal infections), febrifuge, for cough treatment and as a sedative (Rondina et al., 2003; Ratera and Ratera, 1980). In rural areas it is used in feet baths. A twig of the plant placed behind the ear is used for headaches affecting one side of the head (Hieronymus, 1882). In Argentina *L. integrifolia* is an ingredient of some aperitive beverages and teas and also is included in the "Código Alimentario Argentino" (Argentine Food Code). There are only few reports about the chemistry of this species, mainly regarding the composition of its essential oil (Fricke et al., 1999).

Pharmacological reports about *L. integrifolia* extracts can be found in the literature (Coronel et al., 2003; Leitao et al., 2006; Dellacasa et al., 2003; Muschietti et al., 2005; Sülsen et al., 2006). However, to the best of our knowledge, none of them is related to the treatment of gastrointestinal disorders, the most relevant use of this species in traditional medicine. As a part of

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ISSN 0102-695X

our ongoing research, the aim of our study therefore was to evaluate the choleretic and antispasmodic effects of *L. integrifolia* aqueous extract (LIAE) by experimental models in rats.

MATERIAL AND METHODS

Plant material

Aerial parts of *L. integrifolia* (Gris.) Hieronymus (Verbenaceae) were collected in Ampimpa, Tafí del Valle, Tucumán Province, Argentina. The material was identified by Lic. Alberto Slanis and a voucher specimen is deposited at the Herbarium of Fundación Miguel Lillo, under the number 178.

Preparation of the aqueous extract (LIAE)

The aerial parts of *L. integrifolia* (50 g) were air-dried, ground to powder and extracted by maceration with hot water (500 mL) at room temperature for 20 minutes. The extract was filtered and freeze-dried (yield 19.18% w/w of dried plant material).

Drugs

Acetylcholine hydrochloride and sodium dehidrocholate were purchased from Sigma Chemical Co (St. Louis, MO, USA). All reagents used were of analytical grade. Dilutions were made fresh on the day of experiment.

Animals

Female Sprague Dawley rats (210 ± 10 g) were obtained from Animal House of the Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires. They were used taking into account international principles and local regulations concerning the care and use of laboratory animals (Olfert et al., 1993). The animals had free access to a standard commercial diet and water *ad libitum* and were kept in rooms maintained at 22 ± 1°C, humidity 60 ± 5% and a 12 h light/dark cycle.

Choleretic activity

This test was carried out as described by Speroni et al. (2003). Five groups of six rats each were starved for 18 h before the experiment with free access to water and treated by gastric gavages with LIAE dissolved in water at doses of 250, 500 and 750 mg/kg (10 mL/kg) or sodium dehidrocholate (DHC) 500 mg/kg (10 mL/kg) (reference group). Control animals received the same volume of water (10 mL/kg). Immediately, animals were anesthetized with urethane (1.2 g/kg; i.p.). Abdomen was opened by a midline incision and the common bile duct was exposed and cannulated just before the hepatic hilus in order to avoid contamination with pancreatic juice. Rectal temperature was monitored and maintained at 37 ± 0.5 °C throughout the experiment, using a warming lamp. Bile was collected by gravity in pre-weighted vials at 15 min. intervals. Bile flow (BF) was determined by weight and was expressed as µL/min/kg body weight, assuming that the specific gravity of rat bile is 1.0.

Quantitative determination of bile acids

Concentration of bile acids was measured in bile samples. The quantitative determination of the major conjugated and free 3-hydroxy bile acids was performed according to Bruusgaard et al. (1970). The micromethod used a NAD-linked 3 hydroxysteroid dehydrogenase reaction with spectophotometric determination of the resultant NADH.

Antispasmodic activity

Rats were starved for 24 h before the experiment with free access to water. The animals were killed by cervical dislocation without anesthetic agent in order to avoid any influence on the relaxation responses of the tissue.

Jejunums of approximately 1.00 cm in length were prepared and placed in 10 mL organ baths containing Tyrode solution of the following composition (mM): NaCl 135, KCl 5.0, $MgCl_2$ 1.0, $NaHCO_3$ 15.0, NaH_2PO_4 1.0, $CaCl_2$ 2.0, glucose 11.0. Bath solution was maintained at 31 ± 1 ºC and constantly oxygenated with 95% O_2 + 5% CO_2. Tissues were connected to a force displacement transducer for the measurement of isometric force. The preparation was allowed to equilibrate for at least 60 min under 1 g resting tension, and during this period the incubation media was changed every 15 min. In experiments using high KCl solution, the equimolar amount of Na^+ was replaced by K^+ to maintain constant the ion strength. After equilibration, the following experiments were performed.

Effect on dose-response curves to acetylcholine

Cumulative dose-response curves for acetylcholine (Ach) (10^{-9} to 10^{-5} M) were obtained for the tissues. Different concentrations (1.0, 2.0 and 3.0 mg/mL) of LIAE were added to the bath 30 min before constructing the dose-response curve of the agonist.

Effect on dose-response curves to $CaCl_2$

After the initial incubation period, the nutrient solution was replaced by calcium-free hyperpotassic medium (K^+ 75 mM). Cumulative addition of $CaCl_2$ (10^{-4} to $3x10^{-2}$ M) in the absence and presence of different concentrations (0.3, 1.0 and 3.0 mg/mL) of LIAE were

Table 1. Effect of *Lippia integrifolia* aqueous extract (LIAE) (250, 500 and 750 mg/kg p.o.) and DHC (500 mg/kg) on bile flow in rats.

Time (min)	Bile flow (μL/min/kg)				
	Control (H_2O)	LIAE 250 mg/kg	LIAE 500 mg/kg	LIAE 750 mg/kg	DHC 500 mg/kg
15	67.1 ± 3.9	76.3 ± 3.6	107.7 ± 6.2**	98.6 ± 8.3**	137.8 ± 17.3**
30	59.6 ± 2.7	67.4 ± 2.9	92.9 ± 4.2**	96.2 ± 9.4**	104.6 ± 14.9**
45	58.0 ± 3.1	63.5 ± 3.5	85.0 ± 3.0*	68.4 ± 8.2	84.6 ± 10.5
60	53.4 ± 3.8	59.3 ± 3.5	77.5 ± 3.3*	64.5 ± 14.3	74.3 ± 8.3
75	49.6 ± 3.0	56.7 ± 3.6	75.7 ± 3.8*	57.2 ± 16.6	67.1 ± 8.5
90	48.9 ± 2.6	54.4 ± 3.5	71.9 ± 4.7*	56.3 ± 15.0	62.5 ± 8.5
105	46.0 ± 2.9	56.7 ± 6.1	74.6 ± 5.6*	49.6 ± 15.9	66.0 ± 9.5
120	43.8 ± 1.9	59.7 ± 6.9	74.4 ± 6.3*	49.5 ± 15.0	70.4 ± 8.3

Results are expressed as means ± SEM (n = 6), * $p<0.05$, ** $p < 0.01$ versus control group (Student-Newman-Keuls test).

used. Each concentration was in contact for 30 min with the tissue before their effects were evaluated on $CaCl_2$ induced contractions.

Effect on K^+ induced contractions

Jejunum was contracted with KCl (75 mM) which produced a sustained tonic contraction. Different concentrations (0.3, 1.0 and 2.0 mg/mL) of LIAE were added to the bath. In these experiments, the maximal response to the agonist was regarded as 100% (control) and the subsequent response in the presence of the extract was calculated as a percentage of the control response.

Statistics

Pharmacological results are expressed as mean ± SEM. Differences between control and treated groups were tested for significance using a one-way analysis of variance (ANOVA), followed by Student-Newman-Keuls test or Dunnet test. Differences were considered statistically significant when $p < 0.05$ (Graph Pad version 3.1, Graph Soft).

Determination of total caffeoylquinic acids derivatives

The total caffeoyl quinic acids derivatives content of LIAE was measured spectrophotometrically according to Martino et al. (1989). Chlorogenic acid was used as a calibration standard in the range 5 to 16 μg/mL.

RESULTS

Choleretic activity

The effect of orally administered LIAE on rats bile flow is shown in Table 1. Significant increase in BF was obtained after the first 15 min from the oral administration of the extract (500 and 750 mg/kg). The dose of 500 mg/kg induced a significant increase (60.5%) in BF beginning at 15 min after administration, which persisted up to the end of the experiment. No significant increase on BF was observed in animals treated with the dose of 250 mg/kg of LIAE when compared with the control group. At 500 mg/kg the reference drug, DHC, induced a marked but transient increase of BF with 105.3% as a maximum enlargement. Control group presented a slight and regular decrease in BF level during the whole experiment.

Quantitative determination of bile acids

Total bile acids were determined. The oral administration of LIAE induced a significant increase of bile acids output (500 mg/kg: 2655.1 ± 335.2 nmoles/

Table 2. EC_{50} and maximum effect values obtained from the cumulative dose-response curves to Ach and $CaCl_2$ of *Lippia integrifolia* aqueous extract (LIAE).

	Ach			$CaCl_2$	
	EC_{50} (μM)	E_{max} ± SEM		EC_{50} (mM)	E_{max} ± SEM
Control	0.0125 ± 0.004	100	Control	1.04 ± 0.20	100
LIAE 1 mg/mL	0.2372 ± 0.1610*	60.66 ± 1.93**	LIAE 0.3 mg/mL	0.89 ± 0.14	54.52 ± 6.97**
LIAE 2 mg/mL	0.0459 ± 0.0129	67.61 ± 6.74**	LIAE 1 mg/mL	0.86 ± 0.01	32.15 ± 8.5**
LIAE 3 mg/mL	0.1418 ± 0.0846	30.47 ± 3.93**	LIAE 3 mg/mL	1.2 ± 0.12	10.31 ± 2.80**

* $p < 0.05$, ** $p < 0.01$ versus control curve (Dunnet test).

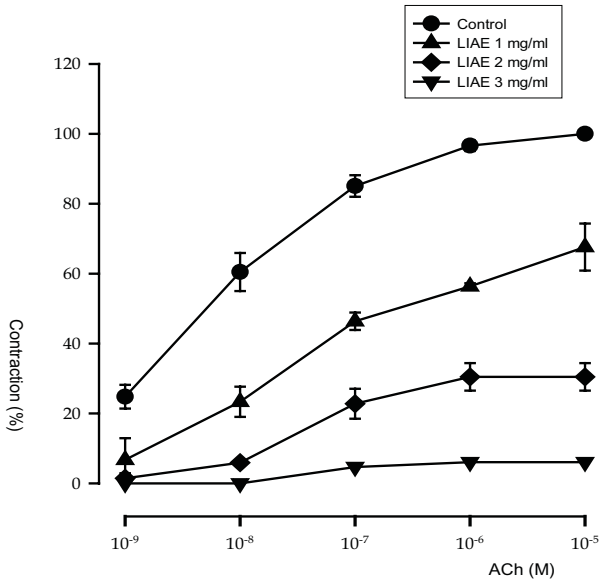

Figure 1. Cumulative log concentration-response curves for Ach in the presence and absence of *Lippia integrifolia* aqueous extract (LIAE). Contractions are expressed as percentages of the maximum control responses. Each point represents the mean ± SEM (n = 5).

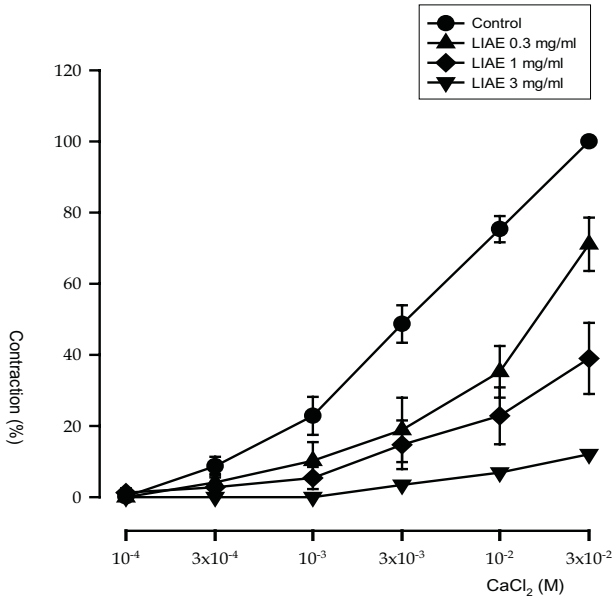

Figure 2. Cumulative log concentration-response curves for $CaCl_2$ in the presence and absence of *Lippia integrifolia* aqueous extract (LIAE). Contractions are expressed as percentages of the maximum control responses. Each point represents the mean ± SEM (n = 5).

min/kg and 750 mg/kg: 2838.8 ± 241.4 nmoles/min/kg) when compared to the control group (1693.2 ± 194.9 nmoles/min/kg).

Antispasmodic activity

LIAE antagonized muscle contractions induced by Ach (1 x 10^{-9} - 1 x 10^{-5} M) and $CaCl_2$ (1 x 10^{-4} - 3 x 10^{-1} M) on rat isolated jejunum (Figures 1 and 2). The extract promoted a dose-dependant flattering of concentration-maximal response curve with Ach and $CaCl_2$ (Table 2). Besides, jejunum contractions induced by 75 mM KCl were also inhibited in a concentration dependant manner by LIAE (0.3 mg/mL: 31.19% ± 4.17, 1 mg/mL: 69.63% ± 6.17, 2 mg/mL: 98.21% ± 1.78).

Determination of total caffeoylquinic acids derivatives

The total caffeoylquinic acid derivatives content in LIAE expressed as chlorogenic acid was 0.10% w/v.

DISCUSSION

A survey of the available literature shows that *Lippia* species are mostly used for the treatment of gastrointestinal and respiratory disorders. However, there are few reports about the pharmacological activities of these species. Most of them have focused their attention on the antimicrobial, antifungal, giardicidal or larvicidal effects (Pascual et al., 2001; Amaral et al., 2006). In this context, we have studied the choleretic and antispasmodic effects of *L. integrifolia*, an Argentine medicinal species, traditionally used for the treatment of digestive complaints, among other uses. Taking into account that decoctions or infusions of the aerial parts of this plant are used in folk medicine, the aqueous extract of *L. integrifolia* was assayed.

The results obtained have clearly shown that LIAE exerts a significant choleretic activity with a significant increase of bile flow and of the bile acids out put. Taking into account that bile secretion is mainly induced through two different mechanisms defined as bile acid-dependent and bile acid-independent secretion, the results of the present study suggest that the bile secretion enhancement of LIAE is bile acid-dependent as shown by the significant increase of bile acids output compared to the control group. These pharmacological effects could be due to the presence of mono and di-caffeoylquinic acids since extensive evidences have reported choleretic and hepatoprotective activities for these compounds (Speroni et al., 2003). Choleretic activity of many medicinal plants has been related to the presence of caffeoyl ester derivates (Handa et al., 1986).

In addition, LIAE showed a significant reduction of the contractions induced by acetylcholine, $CaCl_2$ and KCl on isolated rat jejunum. Gastrointestinal smooth muscle contractions are dependent on intracellular Ca^{2+} concentration, in this sense, two types of excitation-contraction coupling, based on the type of mechanism responsible for changes in Ca^{2+}, are known. High concentration of extracellular KCl induces cell depolarisation and consequently activation of voltage-dependent calcium channels and Ca^{2+} influx into smooth

muscle cell (Al-Zuhair et al., 1996). On the other hand, acetylcholine produces contractions of non-vascular smooth muscle and this effect is mediated by activation of muscarinic receptors causing the release of intracellular calcium stores, without necessarily affecting membrane potential. The results obtained in this study, suggest that the ability of LIAE to attenuate the spasmogenic action of acetylcholine and KCl is unrelated to a specific receptor antagonism. The dose-dependent shifting of the calcium dose -response curve to the right could indicate a possible calcium channel blockade by the extract. LIAE could act on one or several sites in the final common pathway, inhibiting the calcium influx into the cell cytoplasm or interfering with one of the biochemical processes associated with its influx into the smooth muscle cells. Since we deal directly with the total extract, there may be more than one spasmolytic compound involved or the compounds present in the extract may act through different pharmacological mechanisms.

In conclusion, the results obtained suggest that LIAE exerts a significant choleretic activity, with a bile flow enhancement dependent of bile acids excretion and an antispasmodic effect probably produced by inhibition of calcium influx. Further studies must be conducted in the future, for a better understanding of the involucrated mechanisms and the identification of the active compounds responsible for the pharmacological observed effects.

ACKNOWLEDGMENTS

The authors wish to thank SECYT-UBA for financial supporting of this study.

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