Inhibition of Cholinergic Contractions of Rat Ileum by Tropane-Type Alkaloids Present in *Schizanthus hookeri*

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The relative lack of specificity of atropine as a competitive antagonist of muscarinic receptors is a frequent cause of undesirable parasympathetic side effects. Consequently, new tropane alkaloids with potentially greater selectivity are usually seen with real interest. The cholinergic antagonistic effects of a purified mixture of tropane alkaloids extracted from *Schizanthus hookeri* were evaluated in rat ileum. For this purpose, ileal segments were obtained from randomly selected male Sprague-Dawley rats, and the effect of $1 \cdot 10^{-4}$, $1 \cdot 10^{-3}$, and $1 \cdot 10^{-2}$ mg/mL of the purified mixture of alkaloids on the contractile response of the ileum induced with increasing doses of carbachol ($5 \cdot 10^{-8} - 8 \cdot 10^{-4}$ M) was determined. The results were compared with those obtained in the presence of $3.46 \cdot 10^{-7}$, $3.46 \cdot 10^{-6}$, and $3.46 \cdot 10^{-5}$ mg/mL atropine as an agonist. Tropane alkaloids extracted from *Schizanthus hookeri* competitively antagonized acetylcholine muscarinic receptors.

Key words: Tropane Alkaloids, *Schizanthus hookeri*, Cholinergic Antagonism

Introduction

Two well-known natural anticholinergic drugs extracted from plants are atropine and scopolamine usually obtained from the genera *Brugmansia* and *Duboisia* (Bruneton, 2009). These alkaloids exert various effects in the gastrointestinal tract. They decrease stomach peristalsis and tone, thus delaying gastric emptying, decrease intestinal peristalsis and tone, and reduce biliary duct and gall bladder tone, thus diminishing saliva and gastric acid secretion. Their most common clinical use is in pathologies that occur with intestinal colic or spasms, biliary colic or peptic ulcer (Brunton *et al*., 2006).

Similarly to other solanaceous species, species belonging to the genus *Schizanthus* accumulate a wide range of tropane alkaloids (Peña and Muñoz, 2002). This genus includes twelve species native to Chile, with the exception of *S. grahamii* whose area of dispersion includes parts of Argentina (D´Arcy, 1991; Muñoz and Fajardo, 2005; Coccuci, 1989). Some of these species are known under a series of common names such as "poor man's orchid", "little bird flower", and "little bird". Unconfirmed reports indicate that some *Schizanthus* species are used in the Central Chilean Andes by local residents and mountain climbers as stimulants and produce effects similar to those of cocaine (Coccuci, 1989).

Only eight species have been studied, *e.g. S. alpestris*, *S. grahamii*, *S. hookeri*, *S. integrifolius*, *S. littoralis*, *S. pinnatus*, *S. porrigens*, and *S. tricolor* (Muñoz and Fajardo, 2005; Muñoz, 1992; Bieri *et al*., 2006). Previous phytochemical studies revealed that the genus *Schizanthus* R. et P. (Solanaceae) accumulates a large variety of pyrrolidine and tropane alkaloids (Muñoz, 1992; Bieri *et al*., 2006; Cretton *et al*., 2009; Peña and Muñoz, 2002; Lounasmaa and Tamminen, 1993; Humam *et al*., 2008; Jordan *et al*., 2006). The latter are mainly ester derivatives from angelic, senecioic, tiglic,

itaconic, and mesaconic acids. Besides, the presence of dimeric and trimeric tropane alkaloids is another characteristic of this genus. *Schizanthus hookeri* Gill. ex Graham is a plant endemic to Chile. It is an annual herb, 60 cm in height, carrying zygomorphic lilac flowers with a yellow trimmed, deep purple centre and growing in Central Chile (Valparaíso and Santiago regions). It grows in fairly dry regions of altitudes up to 1,300 m. It produces masses of vivid orchid-like flowers and thus is often cultivated for ornamental purposes. Numerous alkaloids have been isolated from this plant, mainly tropane ester derivatives with isomeric C_5 acids (Muñoz and Fajardo, 2005; Humam *et al*., 2007). Among the alkaloids identified from the aerial parts of this species are hygrine, hygrolines A and B, tropine, tropinone, 3*α*,6*β*-dihydroxytropane, tigloidine, cuscohygrine, 6*β*-angeloyloxytropan-3*α*-ol, 6*β*-tigloyloxytropane, schizanthine E, and *N*-methylpyrrolidinylhygrine A or B (Muñoz and Fajardo, 2005; Humam *et al*., 2007; Gambaro *et al.*, 1983).

Most of the drugs of natural origin used as anticholinergics are obtained from the Solanaceae family; they share a common basic structure: the tropane ring. The genus *Schizanthus* presents a varied range of tropane bases, which allows to assume that some of these compounds might have anticholinergic activity or might be precursors that give rise to related biological activity. This encouraged us to investigate the alkaloids present in *Schizanthus* plants as a possible source of new compounds with potentially selective anticholinergic activity.

The presence of tropane alkaloids might endow *Schizanthus* extracts with antispasmodic activity on gastric and intestinal smooth muscle, an effect that can be assessed quantitatively *in vitro* using rat smooth muscle strips, recording changes in tone and spontaneous basal activity. The goal of this study was to determine if the tropane alkaloids present in *S. hookeri* inhibit the tone of ileal smooth muscle through an anticholinergic effect.

Material and Methods

Plant material

The aerial parts of *S. hookeri* Gill. ex Graham were collected in December 2003 in Lagunillas, near Santiago South in the Andes mountain (Farellones, Chile) at 2,100 m above sea level, and identification was confirmed by Prof. Fernanda Pérez, (Departamento de Botánica, Universidad de Chile, Santiago, Chile). A voucher specimen has been deposited at the Facultad de Ciencias Químicas (No. 22231). The plants were dried at ambient temperature for 10 d.

Extraction and analysis

The dried and powdered plant material (1.1 kg dry weight) was extracted successively with *n*-hexane (3 x 2 L) and MeOH (3 x 2 L) at room temperature for 24 h. After filtration, the methanolic solution was evaporated to dryness. The residue (150.2 g) was taken up in 0.1 M HCl and extracted with Et_2O. The aqueous solution was basified with 4% NH_4OH to pH 12 and then extracted with CH_2Cl_2. The organic fraction was dried with anhydrous Na_2SO_4 and the solvent evaporated, yielding 3.1 g of a gummy alkaline residue. Further purification on an aluminium oxide column was performed according to Muñoz (1992).

Alkaloids were identified using gas chromatography coupled to mass spectrometry (GC-MS), by comparison of their retention times, Kovats indices, and fragmentation patterns with those of authentic references. GC-MS was performed in the EI mode at 70 eV, and helium was used as carrier gas at a flow rate of 1 mL/min. Spectra were recorded in the range 30–600 Da at 1.3 scans/s. Injection temperature was set at 250 °C, and the MS transfer line was maintained at 280 °C. The injection was performed in the splitless mode, and the injected volume was 1 μL. Different operating conditions were applied: (i) A 30 m x 0.25 mm i.d. fused silica capillary column coated with the phenyl-methyl silicone phase HP5-MS (0.25 μm film thickness) was used; the temperature program was as follows: isothermal at 40 °C for 2 min, 40–100 °C at 30 °C/min, 100–200 °C at 10 °C/min, 200–300 °C at 5 °C/min, isothermal at 300 °C for 5 min. (ii) A 15 m x 0.25 mm i.d. fused silica capillary column coated with the methyl silicone phase DBI (0.25 μm, film thickness) was used; the temperature program was as follows: isothermal at 45 °C for 2 min, 45–100 °C at 30 °C/min, 100–300 °C at 5 °C/min, isothermal at 300 °C for 5 min.

Animals

Twenty male Sprague-Dawley rats, weighing 200–250 g, were housed under controlled ambient conditions: 12 h light/12 h dark cycle, constant temperature of 22 °C, fed with standardized pellets and water *ad libitum*. They were divided into 2 groups of 10 animals each for the following studies:

1. Determination of the effects of alkaloids extracted from *S. hookeri* on the dose-response curves of ileal strips exposed to carbachol.

2. Determination of atropine effects on the dose-response curves of ileal strips exposed to carbachol.

The rats were anesthetized with 12% (w/v) urethane (1 mL/100 g body weight), and the small intestine was removed. The segment close to the ileo-cecal valve was separated, and fat and adherences were removed. The tissue was placed in a 50-mL isolated organ bath in Tyrode solution containing (in mM): NaCl (136.0), KCl (2.7), $CaCl_2$ (1.8), $MgCl_2$ (1.0), NaH_2PO_4 (0.3), glucose (5.5), and $NaHCO_3$ (12.0), connected to the tension transducer of a digital analog recorder with computer storage, and stabilized at 30 ºC for 40 min under a resting tension of 1 g. A mixture of 95% O_2 and 5% CO_2 was constantly bubbled into the solution. Then, increasing doses of carbachol ($5 \cdot 10^{-8}$ to $8 \cdot 10^{-4}$ M) were added.

Contraction data were adjusted to a sigmoidal dose-response curve using the Graphpad 3.0 program suite. In addition, EC_{50} values and maximal contraction percentages were calculated when appropriate. The effective concentrations (EC) were expressed logarithmically and subjected to a referential parametric analysis (Dunnett's multiple comparison test), with significance set at $P < 0.05$.

Results and Discussion

GC-MS analysis

A preliminary thin-layer chromatography (TLC) examination of the dichloromethane and methanolic extracts of the powdered aerial parts revealed the presence of a large number of alkaloids. Therefore, a simple and reliable GC-MS procedure which has been developed for the identification of tropane alkaloids in *S. grahamii* (Bieri *et al.*, 2006) was applied to the analysis of the alkaloidal mixture. Among them, eight compounds were identified, including hygrine (**1**) and hygrolines A (**2**) and B (**3**) (Fig. 1). These compounds are not discussed in more detail as they are not tropane alkaloids and are frequently found in solanaceous plants. According to the fragmentation patterns, five alkaloids proved to belong to the tropane series. Two isomers of 239 Da were identified by comparison of their retention indices (Humam *et al.*, 2007). The first one was identified as 3α-hydroxy-6β-senecioyloxytropane (**4**) (I = 1866.2) and the second one as 3α-hydroxy-6β-tigloyloxytropane (**5**) (I = 1894.0) (Fig. 1). The tiny quantities of these compounds excluded the possibility of a biological test of their activity. The $[M]^+$ at m/z 365 together with prominent ions at m/z 238, 222, 138, 122, and 94 (base peak) suggested a 3,6-disubstituted tropane nucleus of the molecular formula $C_{19}H_{27}NO_6$ esterified with $C_5H_8O_2$ (tiglic, senecioic or angelic acids) and $C_6H_8O_4$ (methylmesaconic or methylitaconic acids) moieties (**6** and **7** in Fig. 1). However, in the absence of a reference compound, its unambiguous identification was not possible and only tentative assignments were made. This last mixture was used for the biological test.

Effect of the alkaloid mixture on dose-response curves of rat ileal strips exposed to carbachol

Increasing concentrations of alkaloids induced a displacement of the carbachol dose-response curves to the right, and only at $1 \cdot 10^{-2}$ mg/mL, the alkaloid mixture reduced the contractile response significantly compared with controls ($P < 0.05$) (Fig. 2). The contractile response was significantly reduced only with $1 \cdot 10^{-2}$ mg/mL of the alkaloid mixture (Fig. 3). All three concentrations of the *S. hookeri* alkaloid mixture tested caused statistically significant decreases in the EC_{50} value of carbachol as compared with the control ($P < 0.01$, Fig. 4).

Dose-response curves to carbachol in rat ileal strips exposed to atropine

Used as a control of our experimental model, the contractile response induced by increasing concentrations of carbachol was decreased dose-dependently by atropine, but the differences from the controls were not statistically significant ($P > 0.05$, Fig. 5). Likewise, the maximal contractile response elicited by carbachol was not significantly modified by atropine (Fig. 6). The three concentrations of atropine decreased the EC_{50} value, and the changes were significantly different from the controls, with $P < 0.05$ for the low-

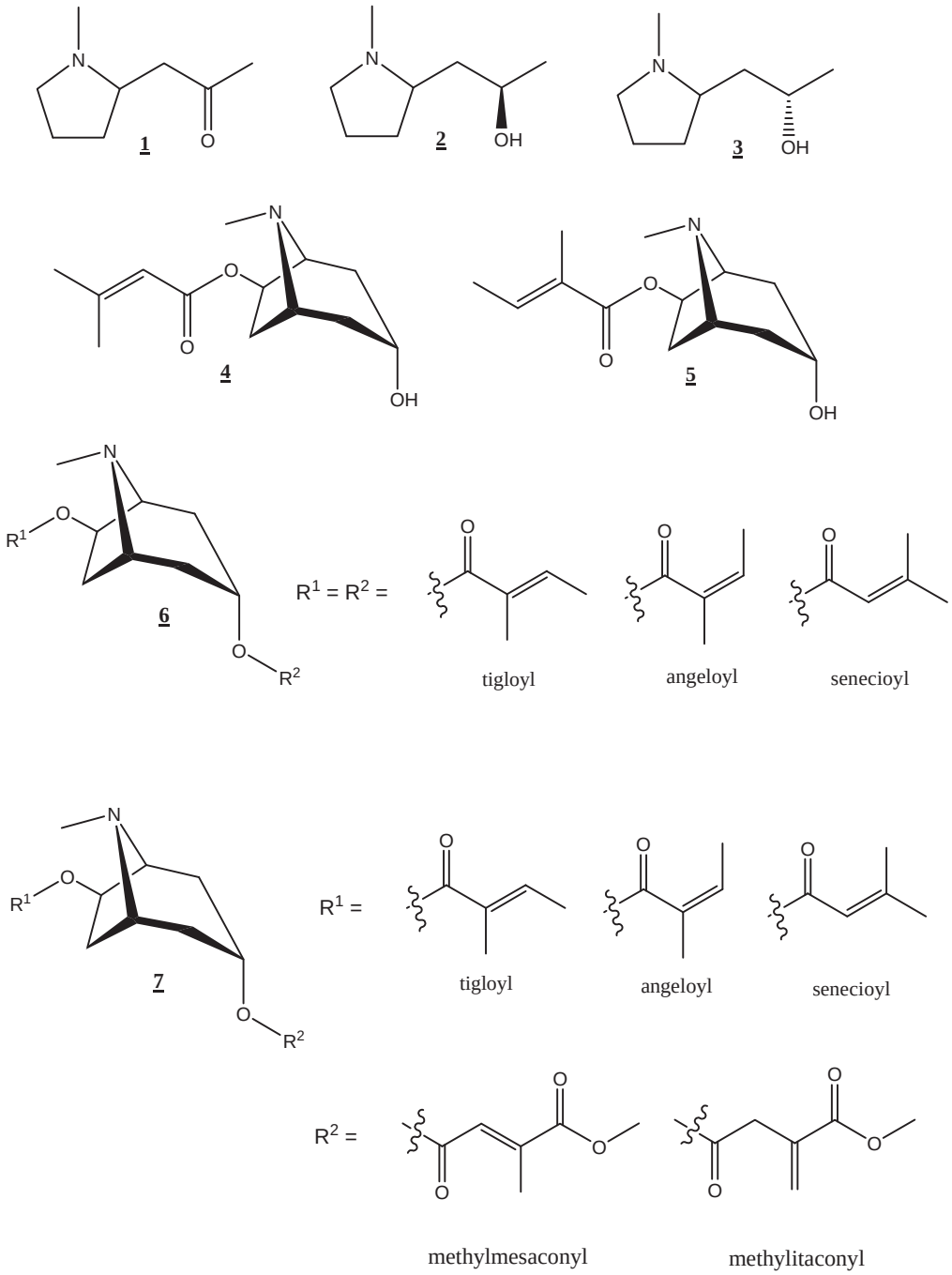

Fig. 1. Chemical structures of the compounds isolated from aerial parts of *Schizanthus hookeri*: hygrine (**1**), hygroline A (**2**), hygroline B (**3**), 3*α*-hydroxy-6*β*-senecioyloxytropane (**4**), 3*α*-hydroxy-6*β*-tigloyloxytropane (**5**). 3,6-Di-substituted tropane nucleus (**6**, **7**) with the indicated acyl moieties.

est concentration and $P < 0.01$ for the two higher ones (Fig. 7).

Based on the obtained results and those reported in the literature, it can be said that the

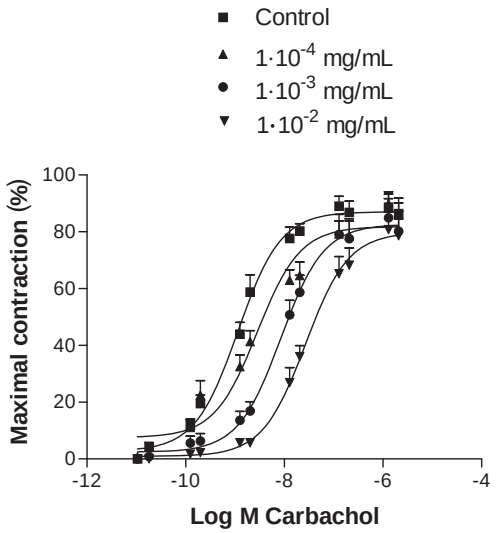

Fig. 2. Dose-response curves for contractions of rat ileal strips exposed to carbachol alone or in the presence of $1 \cdot 10^{-4}$, $1 \cdot 10^{-3}$, and $1 \cdot 10^{-2}$ mg/mL of alkaloids extracted from *S. hookeri*. Each point represents the average ± standard error for tissues from at least 10 animals.

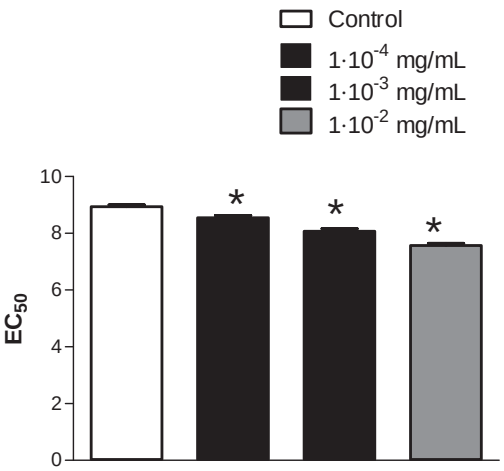

Fig. 4. -Log_{10} EC_{50} in rat ileal strips treated with carbachol alone or in the presence of $1 \cdot 10^{-4}$, $1 \cdot 10^{-3}$, and $1 \cdot 10^{-2}$ mg/mL of alkaloids extracted from *S. hookeri*. Each vertical bar represents the average ± standard error for tissues from at least 10 animals. *$P < 0.01$ compared with controls.

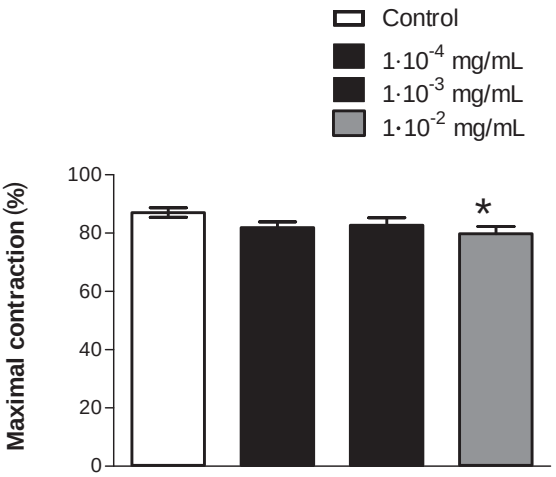

Fig. 3. Contractile response of rat ileal strips to carbachol alone or in the presence of $1 \cdot 10^{-4}$, $1 \cdot 10^{-3}$, and $1 \cdot 10^{-2}$ mg/mL of alkaloids extracted from *S. hookeri*. Each vertical bar represents the average ± standard error for tissues from at least 10 animals. *$P < 0.05$ compared with controls.

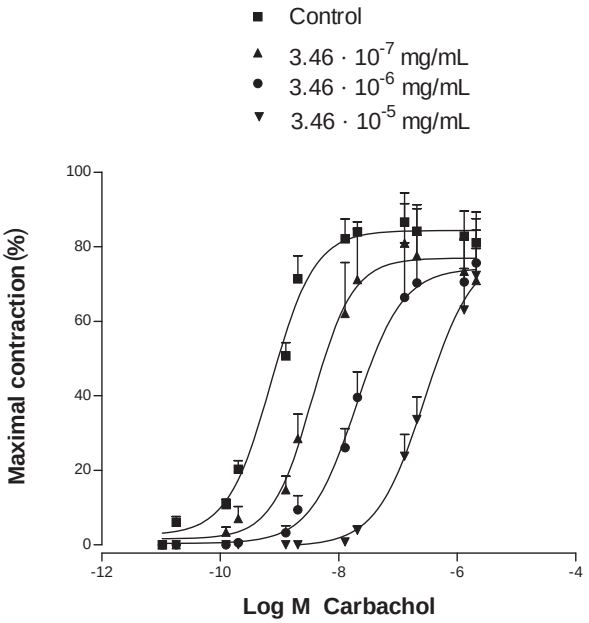

Fig. 5. Dose-response curves for contractions of rat ileal strips exposed to carbachol alone or in the presence of $3.46 \cdot 10^{-7}$, $3.46 \cdot 10^{-6}$, and $3.46 \cdot 10^{-5}$ mg/mL of atropine. Each point represents the average ± standard error for tissues from at least 10 animals.

antagonism exerted by atropine vs. carbachol is a surmountable or competitive inhibition. The application of $1 \cdot 10^{-4}$, $1 \cdot 10^{-3}$, and $1 \cdot 10^{-2}$ mg/mL of *Schizanthus* alkaloid mixture for 10 min prior to the application of cumulative doses of carbachol also caused a shift of the concentration-response curve to the right.

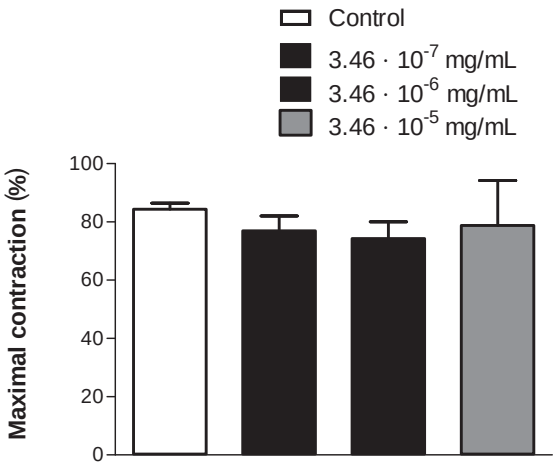

Fig. 6. Contractile response of rat ileal strips to carbachol alone or in the presence of $3.46 \cdot 10^{-7}$, $3.46 \cdot 10^{-6}$, and $3.46 \cdot 10^{-5}$ mg/mL of atropine. Each vertical bar represents the average ± standard error for tissues from at least 10 animals.

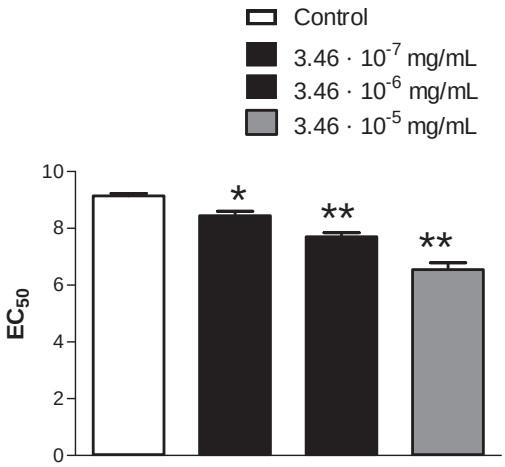

Fig. 7. -Log_{10} EC_{50} in rat ileal strips treated with carbachol alone or in the presence of $3.46 \cdot 10^{-7}$, $3.46 \cdot 10^{-6}$, and $3.46 \cdot 10^{-5}$ mg/mL of atropine. Each vertical bar represents the average ± standard error for tissues from at least 10 animals. $^{*}P < 0.05$ and $^{**}P < 0.01$, respectively, compared with controls.

The EC_{50} values for carbachol alone or in the presence of $1 \cdot 10^{-4}$, $1 \cdot 10^{-3}$, or $1 \cdot 10^{-2}$ mg/mL of *S. hookeri* alkaloid mixture were 1.15, 2.82, 8.51, and $26.91 \cdot 10^{-9}$ M, respectively, and were significantly different with $P < 0.05$. The calculated EC_{50} value for carbachol was significantly lower than the mean value of about $2.0 \cdot 10^{-7}$ M reported in the literature (Glaza *et al.*, 2011). The observed higher potency of carbachol could be explained by an unusual sensitivity to cholinergic agonists in the young specimens of the rat strain used in this study.

The *S. hookeri* alkaloid mixture antagonized the response to carbachol in a competitive fashion: there was a shift to the right in the dose-response curve accompanied by an approximately 2- to 25-fold increase in the EC_{50} value, whereas there was a small, not significant, change in the maximum of the curve. The pattern of antagonism was consistent with a competitive interaction between the *S. hookeri* alkaloid mixture and carbachol, resembling atropine effects on muscarinic receptors in the rat ileal tissue. Using the Schild plot (Furchgott, 1967) to obtain the affinity constant of the *S. hookeri* alkaloid mixture it was estimated to be $5 \cdot 10^{-5}$ mg/mL; and by considering an average molecular weight of the alkaloidal components of about 365 g/mol, it gives an affinity constant, K_B, of $1.37 \cdot 10^{-7}$ M. Similarly, from the experiments with carbachol vs. atropine, the K_B for atropine was estimated to be about $1.34 \cdot 10^{-9}$ M. Thus, the average affinity of the *Schizanthus* alkaloid mixture to the receptor is almost 100 times lower than that of atropine.

Bieri S., Muñoz O., Veuthey J.-L., and Christen P. (2006), Analysis of isomeric tropane alkaloids from *Schizanthus grahamii* by very fast gas chromatography. J. Sep. Sci. **29**, 96 – 102.

Bruneton J. (2009), Pharmacognosie, Phytochimie, Plantes médicinales, 4th ed. Tec & Doc, Paris, France, p. 973.

Brunton L. L., Lazo J. S., and Parker K. L. (2006), The Pharmacological Basis of Therapeutics, 11th ed. McGrawHill, New York, USA, p. 701.

Coccuci A. (1989), El mecanismo floral de *Schizanthus* (Solanaceae). Kurtziana **20**, 113 – 132.

Cretton S., Bartholomeusz T. A., Jeannerat D., Muñoz O., Christen P., and Hostettmann K. (2009), New cyclobutane-containing tropane alkaloids from the aerial parts of *Schizanthus grahamii*. Planta Med. **75**, 916.

D'Arcy W. G. (1991), The Solanaceae since 1976 with a review of its biogeography. In: Solanaceae III: Taxonomy, Chemistry, Evolution (Hawkes J. G., Lester

R., Nee M., and Estrada N., eds.). Royal Botanic Gardens, Kew, Richmond, Surrey, UK, pp. 75 – 137.

Furchgott R. F. (1967), The pharmacological differentiation of adrenergic receptors. Ann. N. Y. Acad. Sci. **139**, 553 – 570.

Gambaro V., Labbe C., and Castillo M. (1983), Angeloyl, tigloyl and senecioyloxytropane. Alkaloids from *Schizanthus hookerii*. Phytochemistry **22**, 1838 – 1839.

Glaza I., Szadujkis-Szadurski L., Szadujkis-Szadurski R., Gajdus M., and Olkowska J. (2011), Modulating activity of M1 receptor to the reaction of ileal smooth muscle. Postepy Hig. Med. Dosw. **65**, 478 – 481(online).

Humam M., Muñóz O., Christen P., and Hostettmann K. (2007), Tropane alkaloids of the aerial parts of *Schizanthus tricolor*. Nat. Prod. Commun. **2**, 743 – 747.

Humam M., Christen P., Muñoz O., Hostettmann K., and Jeannerat D. (2008), Absolute configuration of tropane alkaloids bearing two α,β-unsaturated ester functions using electronic CD spectroscopy: Application to (*R,R*)-*trans*-3-hydroxysenecioyloxytropane. Chirality **20**, 20 – 25.

Jordan M., Humam M., Bieri S., Christen P., Poblete P., and Muñóz O. (2006), *In vitro* shoot and root organogenesis, plant regeneration and production of tropane alkaloids in some species of *Schizanthus*. Phytochemistry **67**, 570 – 578.

Lounasmaa M. and Tamminen T. (1993), The tropane alkaloids. In: The Alkaloids, Vol. 44 (Cordell G. A., ed.). Academic Press, New York, USA, p. 1.

Muñóz O. (ed.) (1992), Solanaceae. In: Química de la Flora de Chile. Ed. Andes y Andes S. A., Santiago, Chile, p. 189.

Muñoz O. and Fajardo V. (eds.) (2005), Flora de Chile. Biología, Farmacología y Química. UPLA Ed., Santiago, Chile.

Peña R. C. and Muñoz O. (2002), Cladistic relationships in the genus *Schizanthus*. Biochem. Syst. Ecol. **30**, 45 – 53.