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Inhibition of acetylcholinesterase activity by hidroalcoholic extract and their fractions of *Bouvardia ternifolia* (Cav.) Shcltdl (Rubiaceae)

[Inhibición de la actividad de acetilcolinesterasa por el extracto hidroalcohólico y sus fracciones de *Bouvardia ternifolia* (Cav.) Shcltdl (Rubiaceae)]

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Abstract
The hydroalcoholic extract (BtHA) and its fractions of *Bouvardia ternifolia* were evaluated as inhibitors of the activity of the acetylcholinesterase enzyme utilizing the *in vitro* method (Ellman). BtHA inhibited the acetylcholinesterase enzyme competitively (IC_{50} = 0.6 μg/ml); the ethyl acetate fraction (BtF-AcOEt) caused mixed-type inhibition (IC_{50} = 0.96 μg/ml). A fraction insoluble on methanol (Bt-Faq-1) showed a mixed-type inhibition (CI_{50} = 0.96 μg/ml). Finally, the methanol-soluble fraction (Bt-Faq-2), presented complex, mixed-type inhibition that corresponds to the C5 system (α = 0.740 and β = 0.842). Rutin, quercetin, kaempferol and ursolic acid were detected by HPLC and the concentration of these compounds was different in each fraction.

Keywords: *Bouvardia ternifolia*, Acetylcholinesterase, quercetin, rutin, kaempferol, ursolic acid.

Resumen
El extracto hidroalcohólico (BtHA) y fracciones provenientes de *Bouvardia ternifolia* fueron evaluadas como inhibidores de la enzima acetilcolinesterasa utilizando el método enzimático propuesto por Ellman. BtHA inhibe a la enzima de manera competitiva (IC_{50} = 0.6 μg/ml); la fracción de acetato de etilo (BtF-AcOEt) provoca una inhibición mixta (IC_{50} = 0.96 μg/ml). La fracción insoluble en metanol (Bt-Faq-1) mostró una inhibición tipo mixta (CI_{50} = 0.96 μg/ml). Finalmente la fracción soluble en metanol, Bt-Faq-2, inhibe a la enzima presentando una inhibición mixta que corresponde a un sistema C5 (α = 0.740 and β = 0.842). Mediante HPLC se detectó rutina, quercetina, canferol y ácido ursólico la concentración de estos compuestos fue diferente en cada fracción.

Palabras Clave: *Bouvardia ternifolia*, Acetilcolinesterasa, quercetina, rutina, canferol, ácido ursólico.

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LISTA DE ABREVIACIONES

AChE = Acetylcholinesterase enzyme; AD = Alzheimer disease; CNS = Central nervous system; FDA = Federal Drug Administration; HPLC = High Performance Liquid chromatography; PNS = Peripheral nervous system; RTs = Retention Times; UV = Ultraviolet.

INTRODUCTION

Due to the chemical complexity of plants and the wide variety of associated biological activities, these are proposed as alternatives in seeking treatments for Alzheimer disease (AD). Some species of the Rubiaceae family have been evaluated for their capacity to inhibit the Acetylcholinesterase enzyme (AChE, EC 3.1.1.7), such as the methanolic extract of *Paederia linearis* Hook (Mukherjee *et al*., 2007), *Warszewiczia coccinea* (Vahl) Klotzsch, whose fractioning led to the isolation of triterpenes with AChE inhibitory activity (Calderon *et al*., 2009). Also, the ethyl acetate extract of *Morinda citrifolia* L. has a neuroprotective effect on inhibiting cognitive deterioration caused by β-amyloid (25-35), in addition to inhibiting AChE (Muralidharan *et al*., 2010).

Bouvardia ternifolia (Cav.) Shcltdl (Rubiaceae) is native to Mesoamerica and Mexico, where it is known as "donita", "candelilla", "hierba del indio", "hierba del pasmo", "trompetilla", "hierba del burro", and "mirto" (Aguilar-Contreras *et al*., 1998; Martínez, 1979). The following are its scientific synonyms: *Bouvardia angustifolia* HBK.; *Bouvardia hirtella* HBK.; *Bouvardia jacquinii* HBK.; *Bouvardia linearis* HBK.; *Bouvardia quaternifolia* HBK.; *Bouvardia tolucana* Hook. et. Arn., *Bouvardia triphylla* Salisb. and *Houstonia coccinea* Andrews (Martinez., 1979).

Peptides have been isolated from this plant such as bouvardin, a cyclic hexapeptide made up of 2 L-alanine, a D-alanine, and 3 modified N-methyl-L-tyrosines, and which possesses a ring composed of the oxidative coupling of the phenolic acid of a tyrosine, by the *ortho* carbon of the phenolic hydroxyl group of an adjacent tyrosine, in addition to its deoxy- and methylated derivatives (Jolad *et al*., 1977). It has been demonstrated that the leaves of the plant contain a high concentration of ursolic and oleanolic acid (Pérez *et al*., 1998). These terpenes possess the following properties: sedative, analgesic, hepatoprotective, anti-inflammatory, antioxidant and AChE-inhibition (Cheng *et al*., 2001), these latter associated with AD. Intraperitoneal (i.p.) administration of the methanolic extract of *B. ternifolia* possesses an antitumor effect *in vivo* and cytotoxic activity on tumor cells of carcinoma *in vitro* at a concentration of 20 μg/ml (Argueta *et al.,* 1994). The hexanic and methanolic extracts presented an inhibitory effect on the toxic action of *Centruroides limpidus limpidus* venom in mice (Jiménez-Ferrer *et al*., 2005). In preliminary studies, it was demonstrated that bouvardin possesses high effectiveness against leukemia P388 and that it is slightly active against melanoma B16; these effects are carried out due to the inhibition of protein synthesis (Johnson *et al*., 1978). It also exerted an effect against the cell division cycle in Chinese hamster cells (Tobey *et al*., 1978).

In the present work, *B. ternifolia* was evaluated as an AChE inhibitor; this species is broadly used in Mexican Traditional Medicine for the treatment of neurological diseases caused by rabies virus infection or by intoxication with scorpion venom. The local population utilizes it for treating "the nerves" (an anthropological disease with symptoms of restlessness, insomnia, appetite loss, cardiac acceleration, and despair), and additionally for treating headache and inflammation (Argueta *et al.*, 1994), the last it is intimately associated with the AD.

Acetylcholinesterase enzyme (AChE, EC 3.1.1.7) plays a preponderate role in the activity of the central (CNS) and peripheral (PNS) nervous systems because it catalyzes the hydrolysis of the Acetylcholine neurotransmitter (ACh) (Legay, 2000). Alzheimer's disease (AD) is a neurodegenerative disease with high incidence worldwide that mainly affects the population aged > 65 years. One of the principal factors that lead to dementia is cholinergic loss or deficiency (Francis, 2006). AChE is one of the main targets of action of drug therapies for this disease (Gandía *et al*., 2006). Some AChE inhibitors such as tacrine, physostigmine, donepezil and rivastigmine comprise some of the treatments that historically have been approved by the Federal Drug Administration (FDA) for the treatment of this disorder (Mimica and Presecki, 2009). Treatment for patients with AD under the AChE inhibition scheme partially delays the symptoms associated with cholinergic neurons such as memory loss, which is the principal alteration of this pathology. Tacrine and physostigmine, the main drugs to be approved for the treatment of the symptoms of AD, have clinical limitations due their low bioavailability and undesirable side effects that

accompany a poor effect on memory (Farlow *et al*., 1992; Watkins *et al*., 1994). On the other hand, second-generation drugs such as donepezil (FDA-approved in 1996) present an adequate pharmacokinetic profile, but also produce side effects such as nausea, vomiting, diarrhea, insomnia, gastrointestinal problems, fatigue, and bradycardia, which can be associated with its cholinergic activity (Roman and Rogers, 2004). In addition to cholinergic loss, this disease has other physiopathological consequences such as inflammation and oxidative stress.

Because of the use of *B. ternifolia* in Mexican Traditional Medicine and its pharmacological and chemical antecedents, in the present work we conducted an evaluation of the anti-AChE activity of the hydroalcoholic extract and its different fractions, as well as its chemical characterization by HPLC.

MATERIALS AND METHODS

Preparation of aerial parts of B. ternifolia and hydroalcoholic extraction

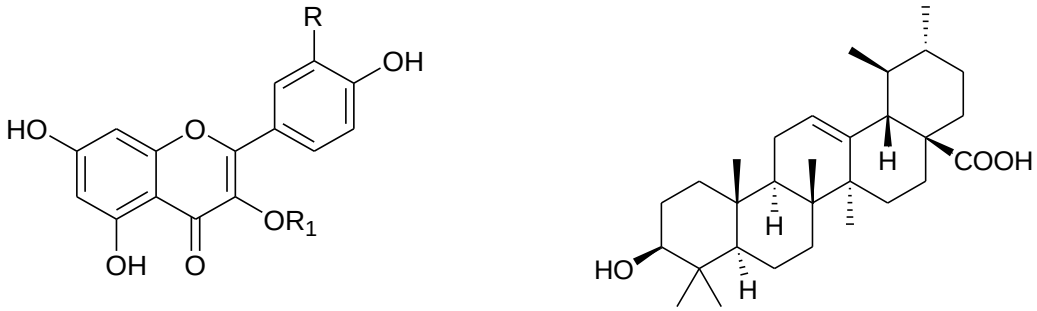
The aerial parts of *B. ternifolia* were collected at (19º 01' 37.94" N; 99º 12' 39.57" W to 2589 msnm) Coajomulco, Huitzilac, Morelos during the period corresponding to September and October 2008. A duly identified sample was deposited at the Centro Medico Siglo XXI Herbarium (IMSSM) and was authenticated by Abigail Aguilar-Contreras, M.Sc., who registered it with the number HPMIMSS 13596. The collected plant material was dried at room temperature under conditions of darkness for 2 weeks. The dry material (870 g) was ground to obtain particles with an average diameter of 4 mm. This plant material was extracted by means of maceration for 2 h with ethanol at 60% at a temperature of 50° C (three times). Later, the extract was filtered on Whatman #1 paper, retaining the plant material to carry out a new extraction under the same conditions. The product gathered from the two extractions was denominated BtHA. Elimination of the solvent was carried out by means of a reduced-pressure distillation process until obtaining a semisolid extract that was finally lyophilized until obtaining a solid extract.

Chemical fractionation of the hydroalcoholic extract

The BtHA (40 g) extract was fractioned through a bipartition process with ethyl acetate/water, obtaining a low-polarity fraction (BtF-AcOEt, organic) and a polar fraction (BtF-Aq, aqueous), which were also concentrated to dryness by distillation and lyophilized. The BtF-Aq fraction was dissolved in methanol to obtain an insoluble amorphous solid and an ochre-colored solution. The precipitate was denominated Bt-Faq-1 and the soluble supernatant was named Bt-Faq-2.

BtHA and its fractions BtF-AcOEt, Bt-Faq-1, and Bt-Faq-2 were utilized for the enzymatic assay; thus, they were stored at l4°C for their later use. Additionally, these were submitted to analysis by HPLC for the chemical characterization based on its content of the triterpene ursolic acid (**1**), as well as of the flavonols quercetin (**2**), rutin (**3**), and kaempferol (**4**) (Figure 1).

Figure 1



	R	R1
Rutin (**1**)	-OH	Rutinose
Quercetin (**2**)	-OH	H
Kaempferol (**3**)	-H	H

Ursolic acid (**4**)

Chemical structure of flavonols and ursolic acid

Chemical analysis of BtHA and fractions

We developed an HPLC (High Performance Liquid chromatography) method for analysis of rutin, quercetin, and kaempferol flavonols using a Waters 2695 separation-module HPLC system equipped with a Waters 996 photodiode array detector and Empower Pro software (Waters Corporation, USA). Flavonols were separated on a supersphere 100 RP-18 column (4 × 125 mm, 5 µm) (Merck, Darmstadt, Germany). The mobile phase consisted of water (pH 3.5, containing 5% TFA) (solvent A) and acetonitrile (solvent B). The gradient system was as follows: 0 - 1 min, 0% B; 2 - 4 min, 10% B; 5 - 7 min, 20% B; 8 - 14 min, 30% B; 15 - 18 min, 40% B; 19 - 22 min, 80% B; 23 - 26 min, 100% B, and 27 - 28 min, 0% B. Flow rate was maintained at 1 mL min^{-1} and the injection volume was 20 µL. Absorbance was measured at 360 nm. Flavonoid peaks were identified by comparison of Retention Times (RTs) and Ultraviolet (UV) spectra with those of commercial reference compounds. The RT for rutin, quercetin and kaempferol was 9.0 min (λ_{max} = 205, 250, and 355 nm), 12.3 min (λ = 205, 250 and 360 nm) and 17.8 min (λ = 205, 250, and 360 nm), respectively. Amounts of flavonols were estimated by interpolation of peak areas and comparison with a calibration curve constructed for each flavonoid. All analyses were repeated by triplicate. Results are expressed as mean values in mg g^{-1} dry extracts.

The same HPLC system and RP-18 column were used for quantification of ursolic acid. In this case, the gradient system was as follows: 0 - 1 min, 0% B; 2 - 3 min, 35% B; 4 - 8 min, 45% B; 9 - 12 min, 70% B; 13 - 14 min, 100% B, and 15 - 18 min, 0% B. Flow rate was maintained at 1 mL min^{-1} and injection volume was 20 µL RT for ursolic acid was 15.6 min (λ_{max} = 191 nm).

Animals

Institute of Cancer Research (ICR) albino mice weighing 30-36 g were employed (Harlan México, DF) and maintained under laboratory conditions at 25º C, with a normal 12 h:12 h light/dark schedule (lights on at 07:00 a.m.) and free access to water and food (pellets from Harlan Rodent Lab Diet). Mice were allowed to adapt to the laboratory environment for 3 weeks before experiments. Experiments were carried out between 8:00 a.m. and 12:00 p.m. All studies were performed in accordance with official Mexican regulation NOM-062-ZOO-1999 (technical specifications for the production, care and use of laboratory animals). The minimum number of animals was employed.

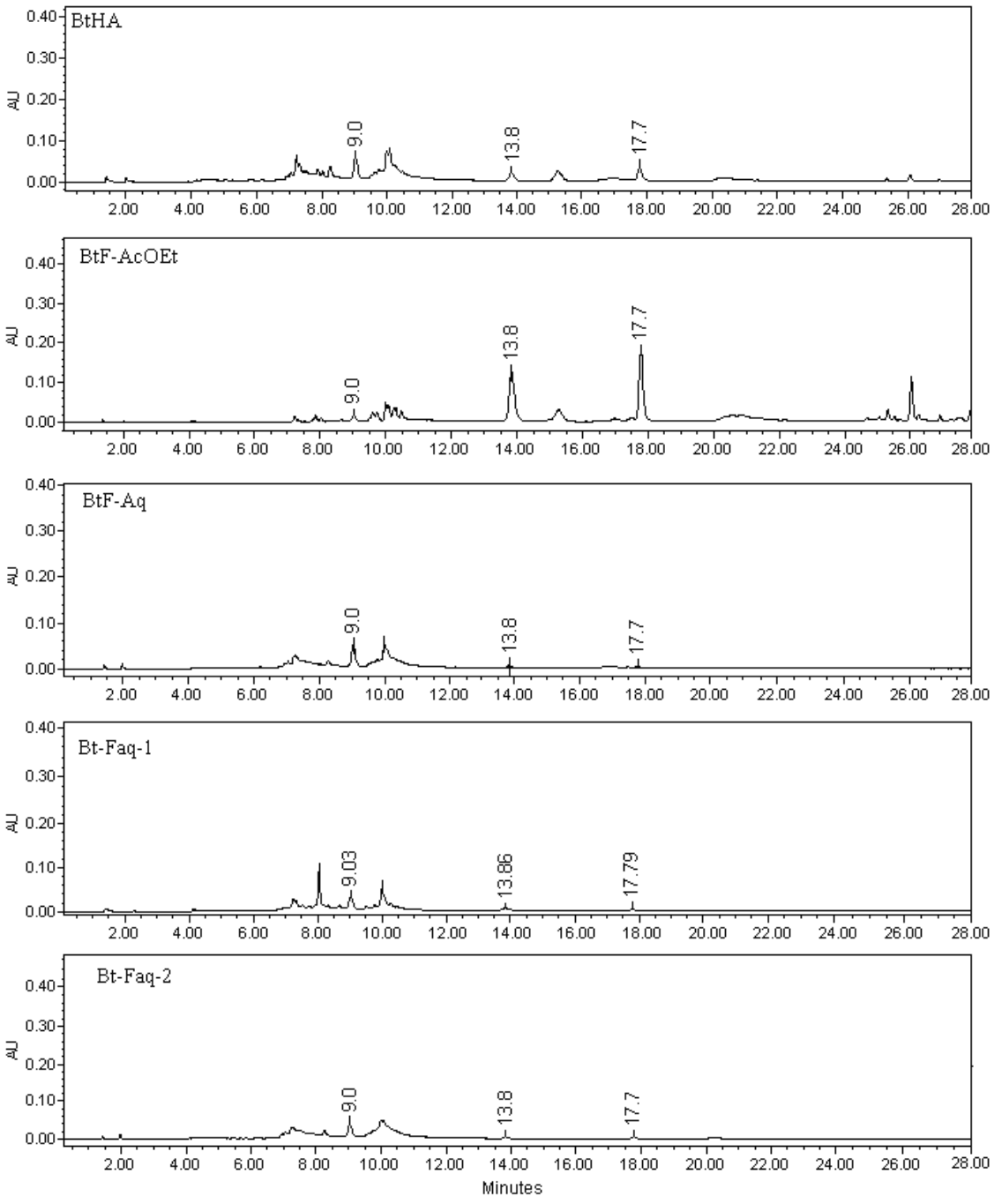
Enzymatic extraction

Four mice were sacrificed by cervical dislocation and the complete brain was obtained immediately. The tissue was suspended in 10 mL of phosphate buffer 150 mM pH 7.2 added to PMFS (phenylmethylsulfonyl fluoride, as inhibitor of proteases) 0.01% and frozen at -70º C for 24 h. Later, the tissue was homogenized in a Potter-type homogenizer. The homogenate tissue suspension was centrifuged at 14,000 rpm for 10 min and the supernatant was utilized as the enzymatic extract, in this extract total protein was quantified to maintain a constant concentration of these in each assay of AChE.

Evaluation of acetylcholinesterase activity in vitro

Evaluation of acetylcholinesterase activity was performed by the method of Ellman *et al*., (1961), in summary, to a final volume of 360 µl we added 300 µl of buffer (100 mM of phosphates pH 8.0), 20 µl of iodide substrate of acetylthiocholine ATChI (Sigma-Aldrich, Mexico) in order to adjust the concentration to 75, 50, 25, 10 and 5 mM; 20 µl of 10 mM of Dithio-bis-nitro-benzoate (DTNB) was added and this was incubated at 37° C. To initiate the reaction, was added 20 µl of enzymatic extract and followed the reaction at 412 nm, reading the changes in absorbance every 20 sec during 3 min. For the inhibition assay, additionally solutions of 0.0125, 0.025, 0.05 mg/ml of BtHA, BtF-AcOEt, Bt-Faq-1and Bt-Faq-2 dissolved in phosphate buffer plus DMSO 5% were utilized. For comparison was used tacrine (1,2,3,4-tetrahydroacridin-9-amine) to 1 x 10^{-5} M.

Figure 2

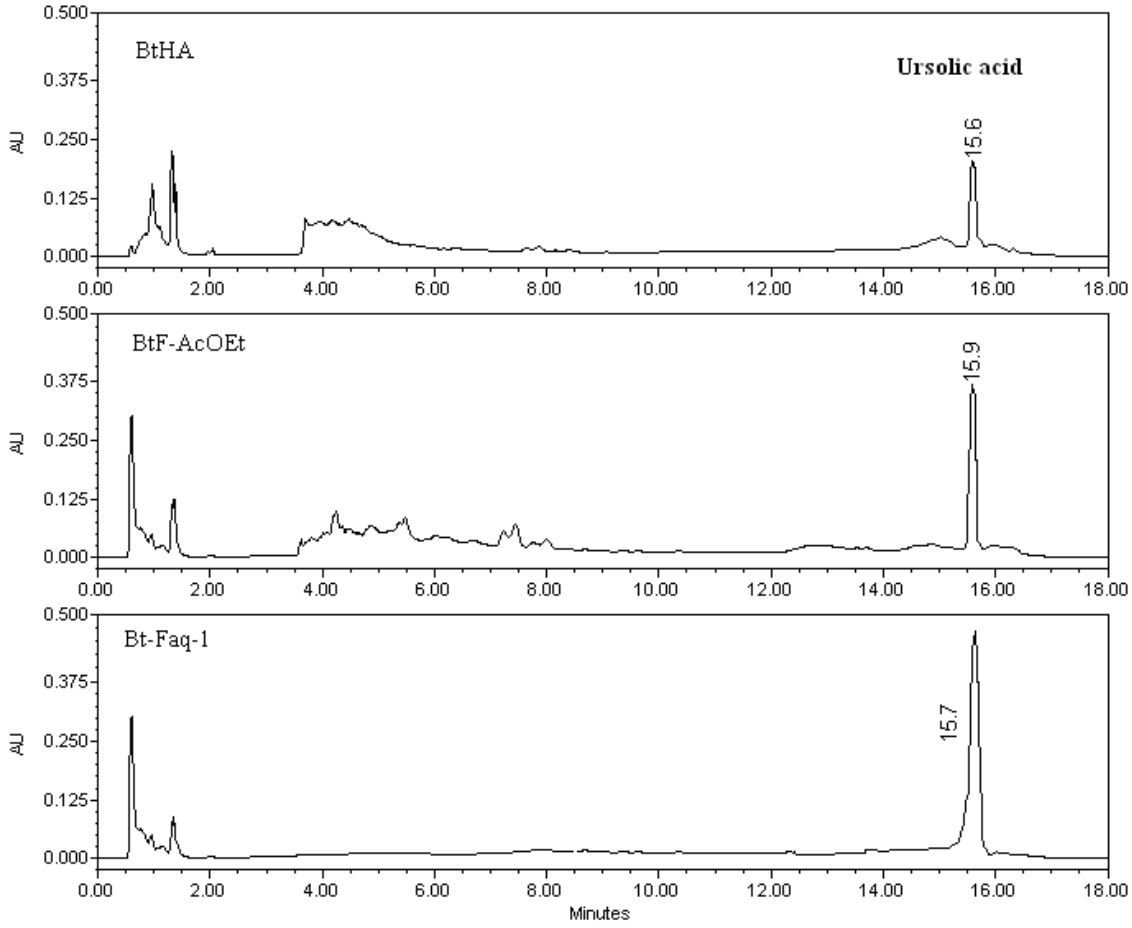


Chromatographic analysis by HPLC of fractions from *B. ternifolia*. BtHA (hydroalcoholic extract), BtF-AcOEt (ethyl acetate/water, organic fraction), BtF-Aq (ethyl acetate/water, aqueous fraction), Bt-Faq-1 (precipitate from resuspension of BtF-Aq) and Bt-Faq-2 (soluble fraction from resuspension of BtF-Aq). The HPLC Method is specific for flavonoids. Rutin T_R = 9.0 min; Quercetin T_R =13.8; kaempferol T_R = 17.7 min

Kinetic enzymatic analysis

To determine enzymatic kinetic parameters, we registered changes in absorbance at 405 nm, determining initial velocity (V_0) as the change in Optical density (OD) in 1 min, at which OD is transformed into the concentration of the product generated utilizing the coefficient of molar extinction of 13,600 (Ellman *et al.*, 1961). The kinetic parameters of AChE activity in the presence of BtHA, BtAcOEt, Bt-Faq-1, and Bt-Faq-2 were analyzed by the plot of Lineweaver-Buck and that of Dixon. In these are represented the reciprocal of the assay's substrate concentration without inhibitor and the growing concentrations of the different extracts. From the same figure, it may be interpreted that the $1/V_0$ axis intercept of the control assay's straight line is $1/V_{max}$ and the slope of the same assay control's straight line is interpreted as K_m/V_{max}. From the graph of the velocity reciprocal vs. the extract's concentration, we were able to obtain the inhibition constant (K_i), which coincides with the point at which the straight line is intercepted by the axis of the extract's concentration; in addition, with the same graph we were able to determine the inhibition type generated by each treatment (Trevor, 1981). The IC_{50} was calculated with the logarithmic transformation graph of the velocity quotient (V/V_0-V), where V_0 corresponds to the velocity in the absence of treatments and V refers to the velocity in the present of treatments (BtHA, BtF-AcOEt, BtF-aq-1, and BtF-aq-2) vs. the logarithm of the extracts' or the fractions' concentration; therefore, the value of IC_{50} corresponded to the concentration of the extract responsible for inhibition at 50% of the enzyme's activity (Giacobini, 1997).

Figure 3



Chromatographic analysis by HPLC of fractions from *B. ternifolia*. BtHA (hydroalcoholic extract), BtF-AcOEt (ethyl acetate/water, organic fraction), Bt-Faq-1 (precipitate from resuspension of BtF-Aq). The HPLC Method is specific for Terpenes: ursolic acid T_R = 15 min

RESULTS

Hydroalcoholic extraction produced 190 g of whole extract (BtHA, 21.8%). Bipartition of 40 g of this extract generated 37.5 g of F-Aq and 2.5 of BtF-AcOEt. Resuspension in methanol of BtF-Aq (20 g) produced 2.5 g of the soluble fraction in methanol (Bt-Faq-1) and 17.5 g of an insoluble precipitate (Bt-Faq-2).

Chromatographic analysis by HPLC of the whole extract and of the fractions of *B. ternifolia* indicated the presence of the following flavonols: rutin (**1**), quercetin (**2**), and kaempferol (**3**). In Figure 2, the chromatogram is depicted, which shows the RTs of these flavonoids: 9.0 min (λ max = 205, 255 and 355 nm), 13.8 min (λ max = 209, 255 and 367 nm), and 17.7 min (λ max = 208, 255 and 367 nm) for rutin, quercetin, and kaempferol, respectively. In Figure 3 the chromatogram shows the presence of ursolic acid with an RT of 15 min and a maximal absorption peak of 191.0 nm.

Quantitative analysis of the flavonoids and the ursolic acid in the distinct treatments allowed determination of the concentration of each chemical compound evaluated. Concentration is presented as mg of the flavonoid or ursolic acid per gram of the dry extract. These amounts are presented in Table 1.

Table 1
Concentration of flavonoids and ursolic acid, in the hydroalcoholic extract and its fractions from *B. ternifolia*

Fractions from *B. ternifolia*	Rutin mg/g Ext	Quercetin mg/g Ext	Kaempferol mg/g Ext	Ursolic acid μg/g Ext
BtHA	19.08	16.89	14.66	6.20
BtF-Aq	17.45	1.88	0.52	Nd
BtF-AcOEt	4.57	100.27	66.98	68.20
Bt-Faq-1	87.00	> 0.003	> 0.003	83.93
Bt-Faq-2	10.53	> 0.003	0.57	Nd

Nd = undetected; Ext = extract; BtHA (hydroalcoholic extract), BtF-AcOEt (ethyl acetate/water, organic fraction), BtF-Aq (ethyl acetate/water, aqueous fraction), Bt-Faq-1 (precipitate from BtF-Aq when was dissolved in methanol) and Bt-Faq-2 (soluble fraction).

Tacrine (9-amine-1,2,3,4-tetrahydroacridine) was utilized as the assay control of inhibition of AChE activity deriving from the mouse brain. In Figure 4 the inhibitor effect of tacrine at a concentration of 1×10^{-5} M is depicted (dotted line), where the transformation of Lineweaver-Buck has an intersection to the left of axis 1/[S]. The tacrine effect was significantly different with respect to that inhibitor group ($p < 0.05$).

The effect of *B. ternifolia* on the activity of the acetylcholinesterase enzyme extracted from mouse brain, was analyzed using different approximations. The former term was employed by the transformation of Lineweaver-Buck (Figure 4). In Panel A of Figure 4, it is shown that the family of straight lines presented a point of intersection in the proximity of $1/V_0$ on the right side. The intercept of the straight line without inhibitor with the axis of p1/[S] indicated the negative inverse value of K_M and the straight lines that represent the effect of the different concentrations of BtHA extract and show the value of apparent K_M (K_{Mapp}); the slope of the straight lines increased the concentration of the inhibitor extract. The effect was significantly different to high concentrations of BtHA respect to data from without inhibitor ($p < 0.05$).

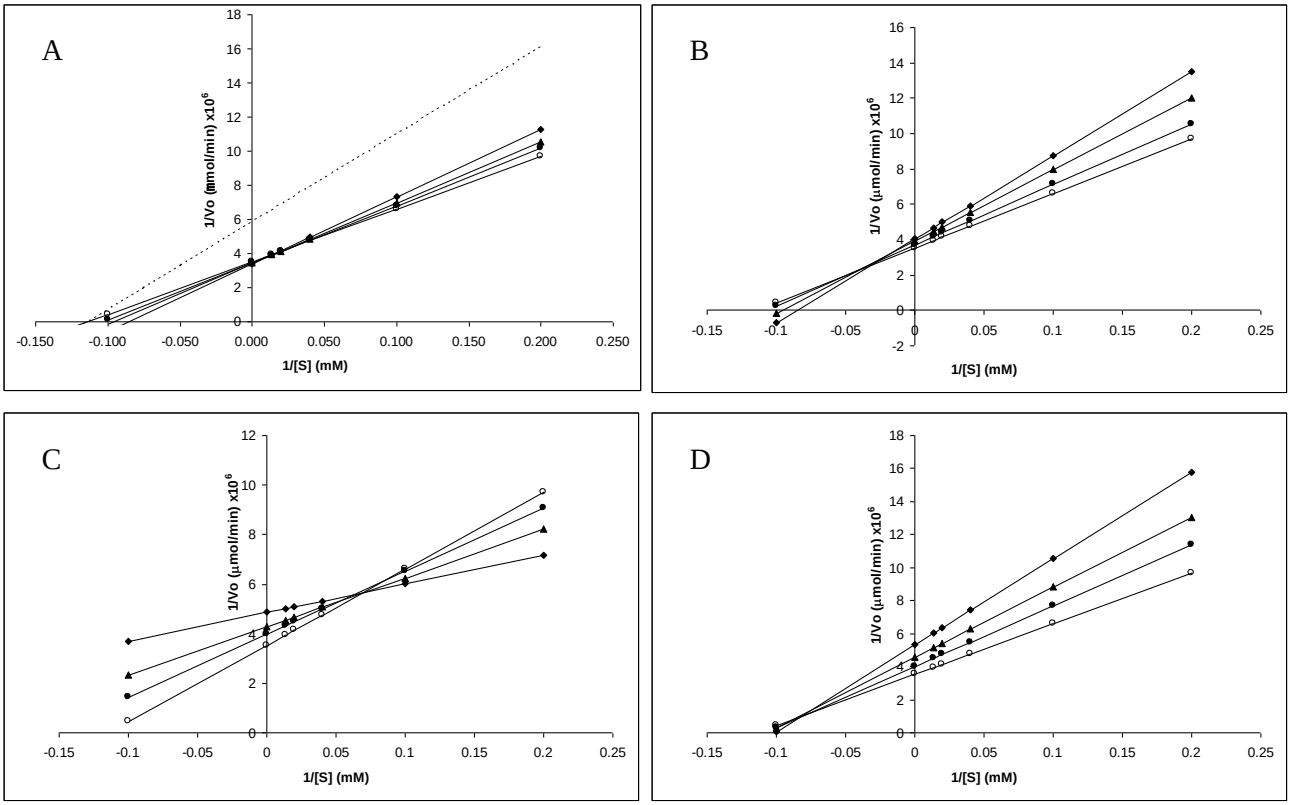
BtF-AcOEt at different concentrations caused the inhibition that is represented in Figure 4B (and this effect was different respect to group without inhibitor, $p < 0.05$), where a family of straight lines is represented that has an intersection point to the left of $1/V_0$ axis above the 1/[S] axis. This behavior corresponds to a mixed inhibition between competitive and non-competitive (Segel, 1975). An increase presented of the slope in the same manner as

the increase in the concentration of the BtF-AcOEt extract.

In Figure 4C, AChE behavior is shown in the presence of increasing concentrations of the soluble methanol extract, Bt-Faq-2. The point of intersection of the family of straight lines is found to the right of the $1/V_0$ axis, where the distance is greater of the point of intersection than that exhibited by the BtHA extract, indicating a mixed-type inhibition corresponding to the C5 system (Segel, 1975), where the slope of the straight lines presents a diminution with respect to the increase of the inhibitor extract concentration, which was statistically different respect without inhibitor ($p < 0.05$).

In Panel D of Figure 4, the inhibitory effect is represented of the precipitate deriving from the methanolic extract Bt-Faq-1, demonstrating a family of straight lines that has a point of intersection to the left of $1/V_0$ and above 1/[S], which corresponds to a mixed inhibition that is intermediate between the competitive and the non-competitive types, similar to that observed with the BtF-AcOEt fraction. The slope of the straight lines increased according to increment of the concentration of Bt-AcOEt, which was significantly different respect without inhibitor ($p < 0.05$).

Figure 4



In Figure 5, the graphs of $1/V_0$ vs. [I] of the different treatments deriving from *B. ternifolia* are presented, in which the families of straight lines are obtained. In Panel A, the effect is presented of the different concentrations of the BtHA extract; the straight lines exhibit a point of intersection that, on being extrapolated to the axis [I], indicated that Ki was 204 μg/ml of BtHA (Cornish-Bowden, 1974).

On testing the BtF-AcOEt and Bt-Faq-1 fractions (Figures 5B and C, respectively), we observed a similar behavior between them, with analogous points of intersection for both straight-line families. The intersection is found in the proximity of the axis [I] and to the right of the origin of $1/V_0$, and the Ki for both extracts was 7.01 and 8.24 μg/ml, respectively.

Figure 6 illustrates the behavior of the straight lines of the graph of $[S]/V_0$ vs. [I]. In Panel A, the effect is presented of BtHA, showing a family of parallel lines, which together with that observed in Figure 4A, where a point of intersection was obtained of the family of straight lines, nearly above the $1/V_0$ axis; Figure 2, where the point of intersection of the straight-line families is found in the quadrant formed by the $1/V_0$ and –[I] axes, indicates a competitive-type inhibition (Cornish-Bowden, 1974). However, because BtHA is a complex composition-type extract, it can be considered that the effect is concerned with the average of the inhibitions shown by the Bt-Faq-1 extract, which is mixed type C5 (Figure 4C) and the Bt-Faq-2 extract, which is of the mixed type between competitive and non-competitive (Figure 4D). Panels B and C of Figure 6 present a similar behavior in which it is confirmed that the effect observed for Figures 4 and 5 and similar to that of Figure 6, for the BtF-AcOEt and Bt-Faq-1 extracts, is concerned with an intermediate, mixed inhibition between competitive and non-competitive behavior (Cornish-Bowden, 1974).

Figure 7 is the graphic representation of $\log_{10}$ $(V/V_0 - V)$ vs. $\log_{10}$ [I]. From the extrapolation of $\log_{10}$ [I] axis to the point of intersection of the straight-line family, we calculated the IC_{50}. For BtHA, we obtained a value of 0.6 μg/ml, as shown in Panel A. In Panels B and C, the behavior is shown of BtF-AcOEt and Bt-Faq-1, respectively; in both cases, the value of IC_{50} was approximately 0.096 μg/ml.

Finally, Table 2 represents a summary of the pharmacokinetic parameters K_M (mM), V_{max} (μmol/min), K_i (μg/ml), and IC_{50} (μg/ml) for each of the experimental conditions assayed.

Table 2
Kinetic constants of Acetylcholinesterase activity from mouse' brain with *B. ternifoli*

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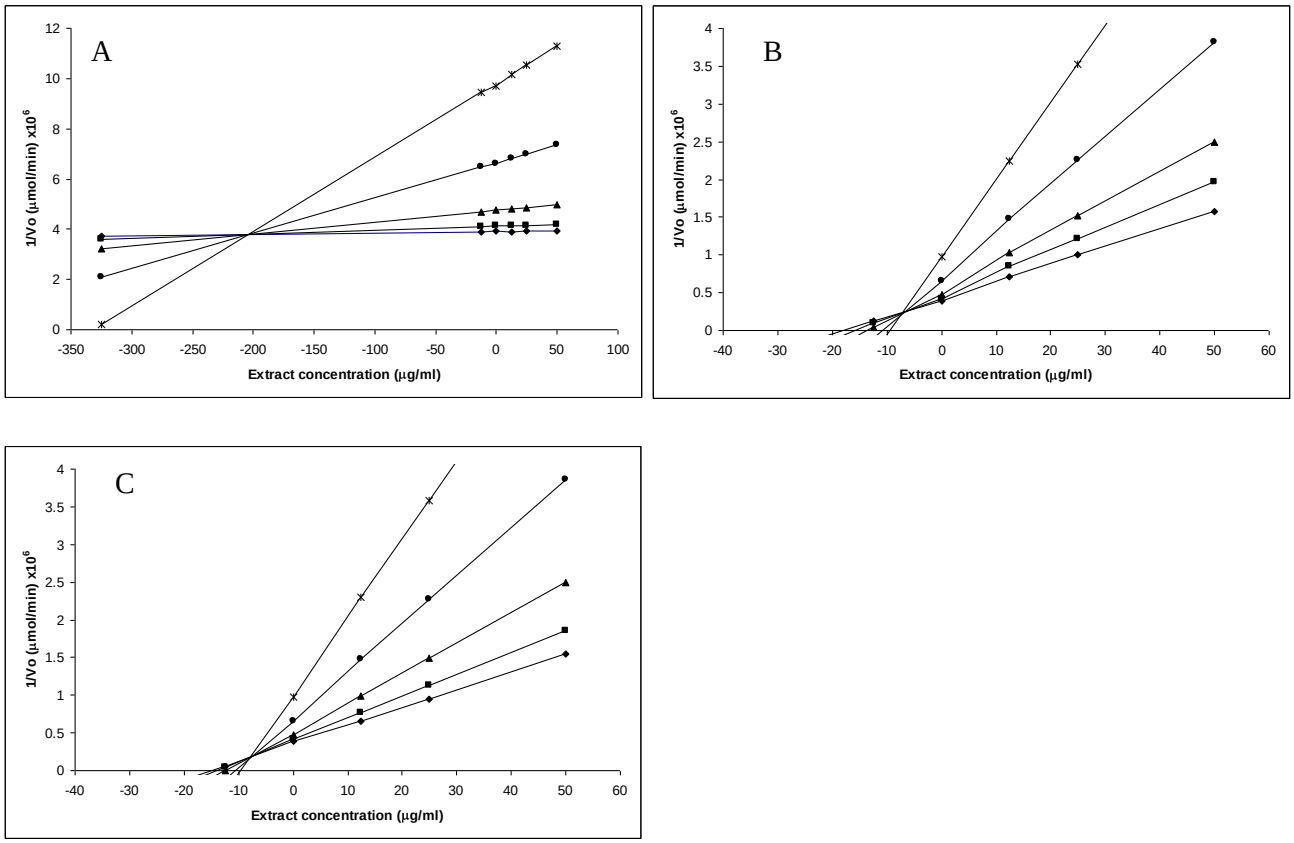
	K_M[(a)] mM	V_{max}[(b)] μmol/min	K_i μg/ml	IC_{50} μg/ml	Inhibition
AchE	8.72	$2.83x10^{-7}$			
BtHA[(c)]	11.58	$2.94x10^{-7}$	204.1	0.60	Competitive**?**
BtF-AcOEt[(c)]	11.56	$2.17x10^{-7}$	7.0	0.096	Mixed*
Bt-Faq-1[(c)]	9.85	$1.88x10^{-7}$	8.2	0.096	Mixed*
Bt-Faq-2[(c)]	2.39	$2.06x10^{-7}$	Nd	Nd	Mixed**
Tacrine	8.7	$1.70x10^{-7}$	1.5***	Nd	No-competitive

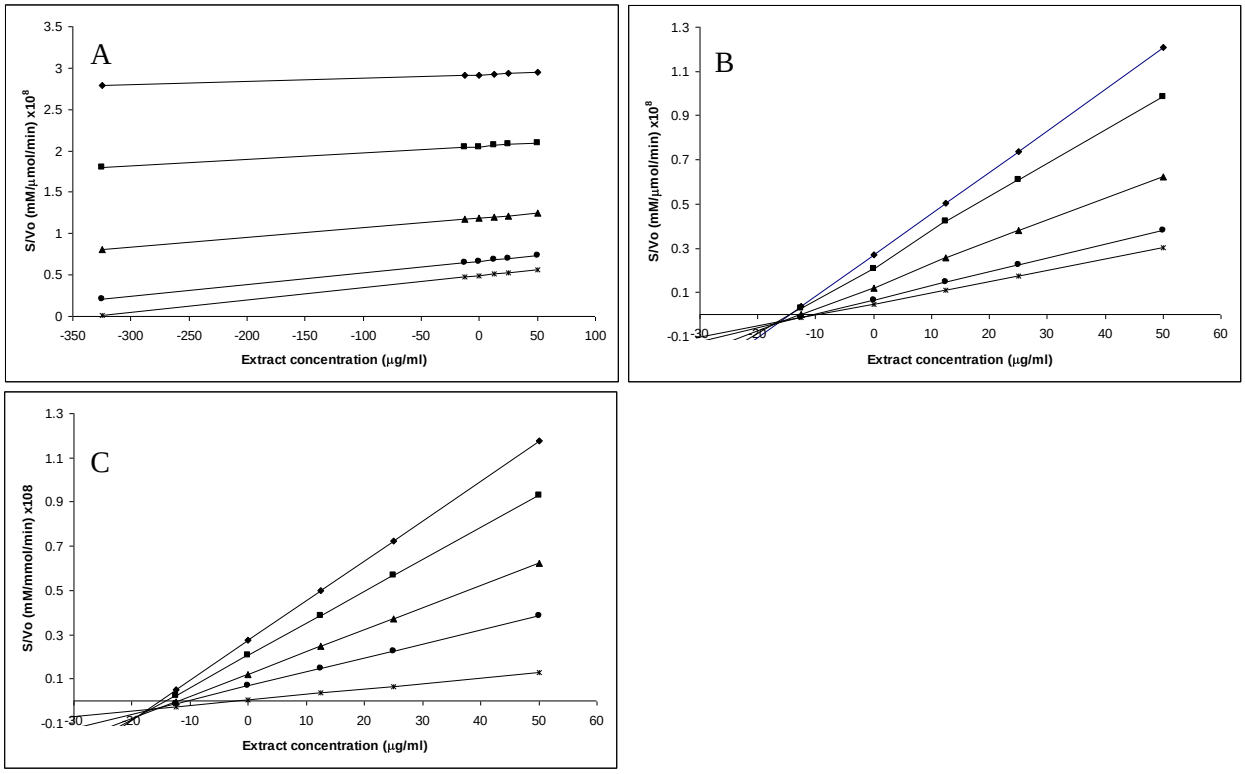
[(a)]The data of K_M in presence of inhibitor is corresponding with to apparent K_M (K_{Mapp}).
[(b)] V_{max} data in presence of inhibitors corresponding to the maximum velocity inhibited V_{maxi}.
[(c)]The concentration used to calculate the constants was 50 μg/ml.
*** Mixed inhibition: intermediate between competitive and non-competitive**
**** Mixed inhibition with values defined for $\alpha = 0.740$ y $\beta = 0.842$**
Nd: undetermined

Figure 5



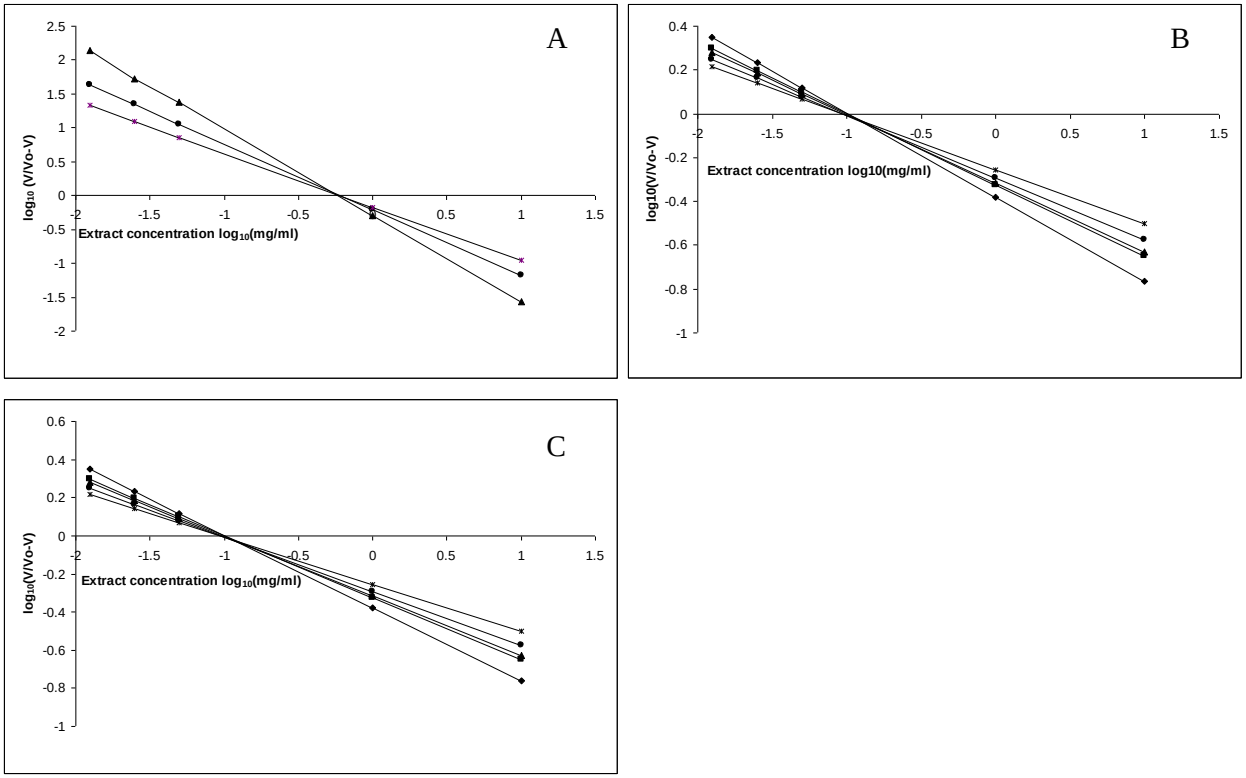
Dixon representation for inhibition of AChE in presence of: A. BtHA (hydroalcoholic extract from *B. ternifolia*); B. BtF-AcOEt (ethyl acetate/water, organic fraction); C. Bt-Faq-1 (precipitate from resuspension of BtF-Aq). With increasing concentrations of substrate Acetyl thiocholine iodide: ◆75 mM, ■ 50 mM, ▲ 25 mM, ● 10 mM, ∗ 5 mM.

Figure 6



Representation of inhibition of AChE, showed [S]/V_o *vs* concentration of treatments. In presence of: A. BtHA (hydroalcoholic extract from *B. ternifolia*); B. BtF-AcOEt (ethyl acetate/water, organic fraction); C. Bt-Faq-1 (precipitate from resuspension of BtF-Aq). With increasing concentrations of substrate Acetyl thiocholine iodide: ◆75 mM, ■ 50 mM, ▲ 25 mM, ● 10 mM, ∗ 5 mM.

Figure 7



Representation of inhibition of AChE, showed $\log_{10}(V/V_o-V)$ *vs* $\log_{10}$ of concentration of extracts (mg/ml). In presence of: A. BtHA (hydroalcoholic extract from *B. ternifolia*); B. BtF-AcOEt (ethyl acetate/water, organic fraction); C. Bt-Faq-1 (precipitate from resuspension of BtF-Aq). With increasing concentrations of substrate Acetyl thiocholine iodide: ◆75 mM, ■ 50 mM, ▲ 25 mM, ● 10 mM, ∗ 5 mM.

DISCUSSION

In this work, we report for the first time the inhibitor effect of the activity of the AChE activity produced by the hydroalcoholic extract of *B. ternifolia*, and of its fractions. The pharmacological characterization of *B. ternifolia* as an acetyl cholinesterase inhibitor arises from the traditional use that the communities have made of this plant, as a protector from the neurological damage associated with different pathologies. The increase of AChE activity is a physiopathological factor related with memory loss, alteration in the speaking capacity and other cognitive affectations in AD. This neurodegenerative disorder is the most common cause of aging-associated dementia and affects 35 million individuals worldwide (Finder, 2010). Targets have been established for the design of drugs for AD treatment, being the most frequently employed drugs the group of substances that inhibit the AChE enzyme. Despite drugs from this group have been approved by the FDA (U.S.), they exhibit important adverse side effects; among the prominent drugs from this group are tacrine (produces hepatotoxicity, nausea, indigestion, vomiting and abdominal pain), donepezil (causes headache, fatigue, dizziness, nausea, vomiting, weight loss, muscle cramping and insomnia), rivastigmine (weight loss, anorexia and dizziness), and galantamine (causes diarrhea, anorexia, cardiovascular disease, heart attack and stroke) (Biran *et al*., 2009), which has lead to the search for medicinal alternatives.

Newman and Cragg (2007), mentioned that 63% of low-weight molecules discovered from 1981-2006 are natural in origin or are compounds derived from natural products. They have been proposed as therapeutic resources for the treatment of alterations derived from the damage caused by AD because of its clinical therapeutic effectiveness as in the case of *Ginkgo biloba* (Gertz and Kiefer, 2004) and its safety and tolerability (Andrieu *et al*., 2003), or due to its well-defined pharmacological targets, as is the case of galantamine (Heinrich and Teoh, 2004). Successful examples of species with anti-AChE activity comprise *Huperzia serrata* (Thunb.) Trevis (Lycopodiaceae), from which huperzine has been isolated that inhibits this enzyme and improves memory (Ortega *et al*., 2004) and *Withania somnifera* (L) Dunal (Solanaceae), from which withanolides with this activity have been isolated (Choudhary *et al*., 2005). Of the Rubiaceae family, the alkaloid extract of *Uncaria rhynchophylla* possesses anti-AChE activity with a value of 10.8 µg/ml (Yang *et al*., 2011). This species also reduces β-amyloid aggregates (Fujiwara *et al*., 2006).

Bouvardia ternifolia (Rubiaceae) presented an AchE inhibition effect which is demonstrated with the analysis of the curves obtained from the reciprocal doubles of Lineweaver-Buck; the behavior of the whole BtHA extract on AChE follows the complex pattern. In the first, analysis of the curved presented in Figure 1 indicates that BtHA exercised a competitive-type inhibition effect, which leads us to think that there are one or several compounds in the complex mixture that is (are) capable of exercising this activity. Analysis by HPLC indicated that this extract possesses a chemical profile that includes the presence of flavonols such as rutin, quercetin and kaempferol, as well as the pentacyclic triterpene, ursolic acid, although the content of the latter in BtHA was very low; thus, it is probable that inhibition of the enzyme caused by this extract is due to the content of flavonoids, and above all, quercetin. It has been demonstrated that quercetin (a commercial reference) exercises a competitive inhibition effect, with a Ki of 3.79 µM with an IC_{50} of 353.86 µM in an assay with AChE deriving from *Torpedo californica*. However, in this same experiment, rutin and kaempferol insignificantly inhibited AChE (Khan *et al*., 2009). In another assay, quercetin at a concentration of 1 mg/ml inhibited 76.2% of the activity of the enzyme AChE (Orhan *et al*., 2007). It has also been demonstrated that this flavonoid isolated from the *Agrimonia pilosa* Ledeb (Rosaceae) presented an IC_{50} of 19.8 µM (Jung and Park, 2007).

Later, fractioning of BtHA with ethyl acetate and water allowed obtaining of the organic fraction (BtF-AcOEt), in which a greater concentration of ursolic acid (68.20 µg/g extract) and of flavonoids (except for rutin) compared with hydroalcoholic acid were identified by HPLC. The BtF-AcOEt fraction, with the mixture of flavonoids and ursolic acid, exerted intermediate, mixed-type inhibition between the competitive and the non-competitive. This inhibition type has been reported for ursolic acid isolated from *Origanum majorana* L (Lamiaceae) in this study, it was shown that this compound possesses the capacity of inhibiting AChE by means of a mixed-type inhibition (between competitive/non-competitive), with a K_i of 6 pM (Cheng *et al*., 2001).

The BtF-Aq fraction was concentrated and resuspended in methanol, obtaining an insoluble fraction in methanol (Bt-Faq-1); this fraction varied considerably with regard to the concentration of flavonoids of which quercetin and kaempferol are barely detected, while rutin is at 87 mg/g of extract. On the other hand, the separation procedure allowed concentration of the fraction toward its content in ursolic acid, which rose to 83.93 µg/g extract. This fraction exerted an inhibitory effect on the intermediate, mixed-type enzyme between competitive and non-competitive, which is in agreement with that reported in the literature for ursolic acid and with data for the BtF-AcOEt fraction. It is probable then that the type of effect exercised by Bt-Faq-1 on the enzyme is conferred mainly by the terpene, due to the other compound found at high concentrations is rutin and, as has already been mentioned, according to the literature, this substance apparently does not exercise significant inhibition on AChE (Khan *et al*., 2009).

Separation of the ethyl acetate fraction was not only chemical but also allowed for dissociation of the type of inhibition exerted by the different groups of compounds; thus, the methanol-soluble fraction (Bt-Faq-2) exhibited mixed behavior in the enzyme, type C5, under the following conditions: $\alpha < 1$; $\beta < 1$, and $\alpha > \beta$. To date, we do not know antecedents in the literature on whether flavonols or terpenes exert this inhibition. The chemical composition of Bt-Faq-2 comprises kaempferol and quercetin in scarcely detectable concentrations (with the analytical method employed) and of rutin of 10 mg/g extract. Ursolic acid was not detected. Probably another type of

compound makes the inhibition behavior to mixed-type inhibition, type C5.

To calculate the inhibition constants of the treatments, we utilized the analysis of Dixon; we observed (Figure 2) how the BtHA fraction led to diminution of the inhibition constant, from 204 µg/ml in the whole extract to 7.01 and 8.24 µg/ml for BtF-AcOEt and Bt-Faq-1, respectively, which indicates that, on diminishing the chemical complexity of the extract, the effect of inhibition is potentialized on the enzyme. The Cornish-Bowden (1974) analysis of Figures 2 and 3 permits us to effect a more detailed description of the behavior of *B. ternifolia*; in this case, we are able to observe that in Figures 2A and 3A, BtHA in effect exerts competitive-type inhibition based on its chemical composition, even if its K_i is high. On the other hand, BtF-AcOEt and Bt-Faq-1 under these conditions also adjust to a mixed inhibitor between competitive and non-competitive. On the other hand, we carried out the calculation of IC_{50} with the Figure 4 graphs; was detected a value of 0.6 µg/ml for the whole extract; this value diminished as the purity of the extracts increased. Due to the similarity in the chemical composition profile between BtF-AcOEt and its fraction, Bt-Faq-1, it is possible to assume that inhibition of AChE in this system is due to the group of low-polarity compounds that includes terpenes such as ursolic acid.

To date, there are no reports on the effects of species of the *Bouvardia* species on neuroprotective processes; however, some plants belonging to the Rubiaceae have shown as antiacetylcholinesterase activity, as in the case of *Warszewiczia coccinea*, whose chemical follow-up led to the structural elucidation of two bioactive triterpenes as inhibitors of AchE: 3β, 6β, 19α-trihydroxyursolic acid, and 3β, 6β-dihydroxyoleanolic acid (sumaresinolic acid) (Calderón *et al*., 2009). It is feasible to assume that the inhibition effect caused by *B. ternifolia* is due to its high content of ursolic and oleanolic terpenes, from which these have separated 80 mg from the former and 50 mg from the latter, under a column-chromatography fractioning scheme that utilized ethyl-acetate concentration gradients: chloroform (Pérez *et al*., 1998). Bearing in mind that this plant is utilized against inflammatory processes, which in addition is accompanied by the demonstration (in this work) of anti-AChE activity and the verification that within its chemical constitution is found ursolic acid with a mixed inhibitory effect on AChE (Cheng *et al*., 2001), but in addition possesses important anti-inflammatory properties together with oleanolic and betulinic acid inhibiting the liberation of Interleukin-6 (IL-6) of a culture of Mono Mac 6 cells (Sabih *et al*., 2011) and also is capable of inhibiting 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion in skin (Tozuda *et al*., 1986). In fact, preliminary studies on the BtHA extract of the aerial parts of this species induced an anti-inflammatory effect in the TPA-induced auricular edema model and diminution of cognitive deterioration in mice which received scopolamine in the T-form maze test (data not shown, http://www.relaquim.com/archive/2008/memorias_5_reunion_%20prod_nat.pdf).

CONCLUSION

According to the cholinergic hypothesis and that AChE inhibitors improve neuronal transmission prolonging the effect of acetylcholine, and with this, the temporary diminution of cognitive deterioration, *B. ternifolia* is therefore presented as a plant candidate for continuing its study as a potential source of compound usefully against the Alzheimer's disease.

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