CHAPTER III

RESEARCH METHODOLOGY

Animals

ICR mouse 28-32 g from National Laboratory Animal Center, Mahidol University, Nakhon Pathom, Thailand.

Materials

1. Trizma® hydrochloride minimum 99% titration (Sigma-Aldrich, Inc., St. Louis, MO, USA)
2. Ethylenediaminetetraacetic acid disodium salt (Sigma-Aldrich, Inc., St. Louis, MO, USA)
3. Sodium dodecyl sulfate (Biobasic, Inc., ON, CA)
4. Sodium deoxycholate (Sigma-Aldrich, Inc., St. Louis, MO, USA)
5. Sodium orthovanadate, minimum 90% titration (Sigma-Aldrich, Inc., St. Louis, MO, USA)
6. Sodium chloride (Merck, Darmstadt, Germany)
7. Triton X-100, General purpose grade (Fisher scientific UK Limited, Leicestershire, UK)
8. Phenylmethylsulfonyl fluoride (Sigma chemical company, St. Louis, MO, USA)
9. Sodium pyrophosphate dibasic, practical grade (Sigma-Aldrich, Inc., St. Louis, MO, USA)
10. Ammonium persulfate, for electrophoresis, ≥98%, (Sigma-Aldrich, Inc., St. Louis, MO, USA)
11. Tris-base, minimum 99%, (Sigma-Aldrich, Inc., St. Louis, MO, USA)
12. 3-(Cyclohexylamino)-1-propanesulfonic acid, (Sigma-Aldrich, Inc., St. Louis, MO, USA)
13. Glycine SigmaUltra, >99% titration, (Sigma-Aldrich, Inc., St. Louis, MO, USA)

14. UltraPure™ glycerol (Invitrogen life technologies, CA, USA)

15. Methanol (RCI Labscan Ltd, Bangkok, Thailand)

16. Acetone (RCI Labscan Ltd, Bangkok, Thailand)

17. Propan-1-ol (RCI Labscan Ltd, Bangkok, Thailand)

18. Bromophenol blue, (Sigma-Aldrich, Inc., St. Louis, MO, USA)

19. Tween® 20 (BDH, England)

20. Skimmilk (Merck, Darmstadt, Germany)

21. 2-Mercaptoethanol, electrophoresis purity reagent (BIO-RAD Laboratories, Inc., CA, USA)

22. N, N, N', N'- Tetramethylethylenediamine (TEMED) (BIO-RAD Laboratories, Inc., CA, USA)

23. 40% Acylamide/Bis solution 37.5:1 (2.6%C) (BIO-RAD Laboratories, Inc., CA, USA)

24. Polyvinylidene fluoride (PVDF) membrane (Pall Corporation, FL, USA)

25. Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific, Inc., IL, USA)

26. Pierce® ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc., IL, USA)

27. Protease inhibitors cocktail (Sigma-Aldrich, Inc., St. Louis, MO, USA)

28. Full-range Rainbow Molecular weight Markers (GE Healthcare Bio-Sciences AB, Uppsala, Sweden)

29. Developer and replenisher (Carestream Health, Inc., NY, USA)

30. Fixer and replenisher (Carestream Health, Inc., NY, USA)

31. Normal saline 0.9% (General Products Public Co., Ltd., Pathum Thani, Thailand)

32. (-)-Nicotine hydrogen tartrate salt, ≥98% (TLC) (Sigma-Aldrich, Inc., St. Louis, MO, USA)

33. Mecamylamine hydrochloride (Sigma-Aldrich, Inc., St. Louis, MO, USA)

34. Sodium phosphate monobasic, SigmaUltra, minimum 99.0% (Sigma-Aldrich, Inc., St. Louis, MO, USA)

35. di-Sodium hydrogen orthophosphate anhydrous (Ajax Finechem Pty, Ltd., NSW, AU)

36. Potassium chloride (Ajax Finechem Pty, Ltd., NSW, AU)

37. Calcium chloride (Ajax Finechem Pty, Ltd., NSW, AU)

38. Magnesium chloride anhydrous (Sigma-Aldrich Chemie GmbH, Germany)

39. 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), minimum 99.5% titration (Sigma-Aldrich, Inc., St. Louis, MO, USA)

40. Atropine, minimum 99% TLC (Sigma-Aldrich, Inc., St. Louis, MO, USA)

41. Carbamoylcholine chloride, minimum 98% TLC (Sigma-Aldrich, Inc., St. Louis, MO, USA)

42. Microscint™ scintillation cocktail (PerkinElmer, Inc., MA, USA)

43. nAChRα7 (H-302): sc-5544 (Santa Cruz Biotechnology, Inc., CA, USA)

44. mAChRM5 (H-197): sc-9110 (Santa Cruz Biotechnology, Inc., CA, USA)

45. Actin (C-2): sc-8432 (Santa Cruz Biotechnology, Inc., CA, USA)

46. (3-Aminopropyl)trimethoxysilane, 97% (Sigma-Aldrich, Inc., St. Louis, MO, USA)

47. Kodak BioMax Transcreen-LE, 20.3x25.4 cm. (CARESTREAM HEALTH, Inc., NY, USA)

48. Kodak BioMax MS Film, 20.3x25.4 cm. (CARESTREAM HEALTH, Inc., NY, USA)

49. [^{3}H]-nicotine (Amersham Biosciences UK Limited, Buckinghamshire, UK)

50. [^{3}H]-scopolamine (Amersham Biosciences UK Limited, Buckinghamshire, UK)

51. Autoradiographic [^{3}H] micro-scales, 110 nCi/mg (0.111-4.07 kBq/mg). (Amersham Biosciences UK Limited, Buckinghamshire, UK)

Instruments

1. Multimode Detectors, DTX-880 (Beckman Coulter, Inc., CA, USA)
2. Camcorder, Sony handycam® (Sony Corporation, Tokyo, Japan)
3. Quantity One®, 1-D electrophoresis gels analyzing software dot blots, Bio-Rad, CA, USA.
4. TopCount NXT™ Microplate Scintillation and Luminescence Counter (PerkinElmer, Inc., MA, USA)
5. The FilterMate Universal Harvester (PerkinElmer, Inc., MA, USA)
6. UniFilter-96 GF/B, White 96-well Barex Microplate with GF/B filter of 1 µm poresize (PerkinElmer, Inc., MA, USA)
7. Elevated plus maze (Faculty of Pharmaceutical sciences, Phitsanulok, Thailand)
8. Open-field apparatus (Faculty of Pharmaceutical sciences, Phitsanulok, Thailand)
9. Mini protean® III cell (BIO-RAD Laboratories, Inc., CA, USA)
10. Mini protean® trans-blot (BIO-RAD Laboratories, Inc., CA, USA)
11. Leica CM 1850 cryostat (Leica Microsystems Inc., IL , USA)

Experimental procedures

1. Plant extracts preparation

The flower and leaf of *V. cinerea* were obtained from Bangkratum hospital, Phitsanulok, Thailand. One hundred grams of dried crushed of *V. cinerea* were macerated with 1 liter of 60 °C distilled water for 24 hours. The aqueous extracts were subsequently filtered and allocated 15 ml of the aqueous extract to each 20 ml glass bottle. The extracts were stored at -20 °C at least 24 hours before dried with freeze-drying apparatus for 4 days. The extracts powders were kept at -20 °C. The percent yield of the extract was calculated using the following equation:

% yield = [dried extract weight (g) / plant material sample weight (g)] x 100

2. Animals

The experiments were performed using ICR mouse (National Laboratory Animal Centre, Mahidol University, Nakhon Pathom, Thailand.) with weights of 28-32 g at the beginning of the experiments. The animals were maintained under standard laboratory conditions (25 °C and 12 hours light/dark cycle, with free access to food and drinking water). Animal handling and experiments were approved by the Ethical Committee for the Use of Animal, Naresuan University. Each experimental group consisted of 6 animals. Animals were randomly allocated to different groups as the following: control group (C), nicotine withdrawal group (NW), group received mecamylamine after nicotine withdrawal (MEC), group received VE 125 mg/kg after nicotine withdrawal (VE125), group received VE 250 mg/kg after nicotine withdrawal (VE250), and group received VE 500 mg/kg after nicotine withdrawal (VE500), as shown in Table 2.

Table 2 Animals treatments

Groups	Treatments	
	Day 1-14 (Nicotine treatment period)	Day 15-21 (Nicotine withdrawal period)
Control (C)	Normal saline, s.c.	Normal saline, i.p.
Nicotine withdrawal (NW)	2 mg/kg of nicotine, s.c.	Normal saline, i.p.
Mecamylamine (MEC)	2 mg/kg of nicotine, s.c.	2 mg/kg of MEC, i.p.
V. cinerea 125 mg/kg (VE125)	2 mg/kg of nicotine, s.c.	125 mg/kg of VE, i.p.
V. cinerea 250 mg/kg (VE250)	2 mg/kg of nicotine, s.c.	250 mg/kg of VE, i.p.
V. cinerea 500 mg/kg (VE500)	2 mg/kg of nicotine, s.c.	500 mg/kg of VE, i.p.

Note: Abbreviations s.c.; subcutaneous, i.p.; intraperitoneal

3. Treatments

V. cinerea extracts were dissolved in 0.9% normal saline to three final concentrations of 10, 20, and 40 mg/ml. Mecamylamine (Sigma-Aldrich, Inc., St. Louis, MO, USA) was dissolved in 0.9% normal saline to final concentration of 1.875 mg/ml. Nicotine hydrogen tartrate salt (Sigma-Aldrich) was dissolved in 0.9% normal saline to final concentration of 1.875 mg/ml. All of stock solutions have been allocated 1.0 ml to each micro centrifuge tube and stored in -20 °C until use.

For nicotine treatment period, 2 mg/kg of nicotine was administered subcutaneously with 26 G ½" hypodermic needle (0.45x12 mm.) to five groups of mice (NW, MEC, VE125, VE250, and VE500) four injections daily with 4 hours apart, starting from 08.00 a.m. for 14 days to induce withdrawal symptoms [61].

For nicotine withdrawal period (day 15 to 21), immediate injection of substance were performed on day 15 as shown in Table 2. A maximal injection volume was set to 0.15 ml.

4. Behavioral tests

4.1 Evaluation for abstinence signs

Prior to each observation session, mice were placed in clear cages to habituate for 30 minutes, and they were returned to their home cage upon completion of the behavioral evaluation. Abstinence signs were evaluated for 30 minutes in clear cages after first (day 15) and last days of nicotine withdrawal (day 21). For the evaluation of the behavioral signs, The Nicotine Abstinence Scale was compiled and utilized to score the frequency of the following signs: rearing, body lifting, abdominal constrictions, nose scratching, ear scratching, dog shaking, body shaking, body scratching, and chewing during 30 minutes of observation [61] (Figure 4).

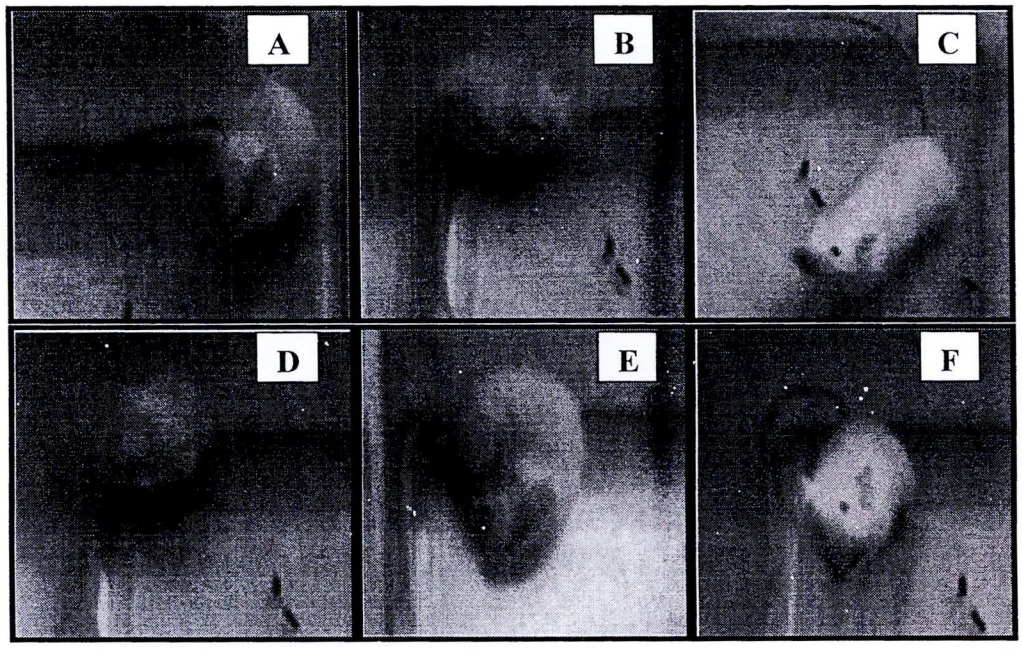

Figure 4 Some abstinence signs in mice including nose scratching (A), ear scratching (B), body lifting (C), body scratching (D), dog shaking (E), and rearing (F)

4.2 Evaluation of anxiety-like behavior

Anxiety responses were measured in the elevated plus maze (EPM) test [71, 72]. The experimental apparatus was shaped like a "plus" sign and consisted of a central platform (5×5 cm), two open arms (30×5 cm) and two equal-sized closed (30×5×15 cm) arms opposite to each other. The maze was made of black metal sheet, elevated to a height of 50 cm above the floor (Figure 5). The test consisted of placing a mouse in the central platform facing an enclosed arm and allowed it to freely explore the maze for 5 minutes. Entry into one arm was defined as the animal placing all four paws into that arm. The test arena was cleaned with 70% alcohol after each trial. The number of total entries and the time spent in open arms were measured by an observer blind to the drug treatment. Total entries and percent time spent in open arms were used as a measure of anxiety-like behavior due to the behavior of mice that usually fear and avoid unprotected area of novel environment, and bright field.

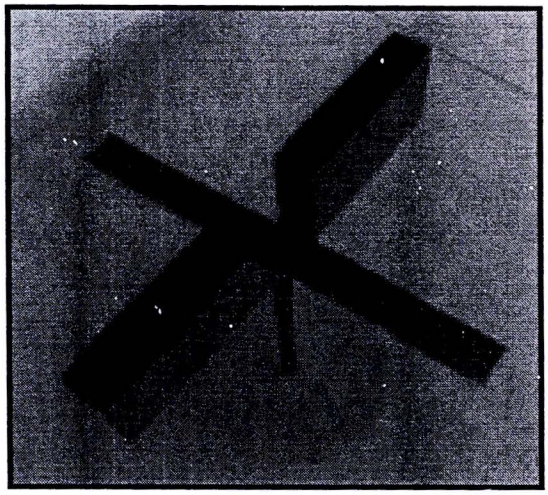

Figure 5 Elevated plus maze apparatus.

4.3 Locomotors activity (LMA)

Normally, hyperlocomotor activity of mouse can occur by nicotine withdrawal. Locomotor activities were measured with an open-field apparatus [72]. The apparatus was surrounded with black metal sheets (50x50x30 cm). The bottom of this apparatus was divided into 25 blocks with 10x10 cm per block (Figure 6). The experiment was commenced with placing a mouse to the center of the apparatus and allowed it to freely explore. Ambulatory locomotion of the animal was scored a number of squares entered within 5 minutes. Square enter was defined as the animal placing all four paws into that square.

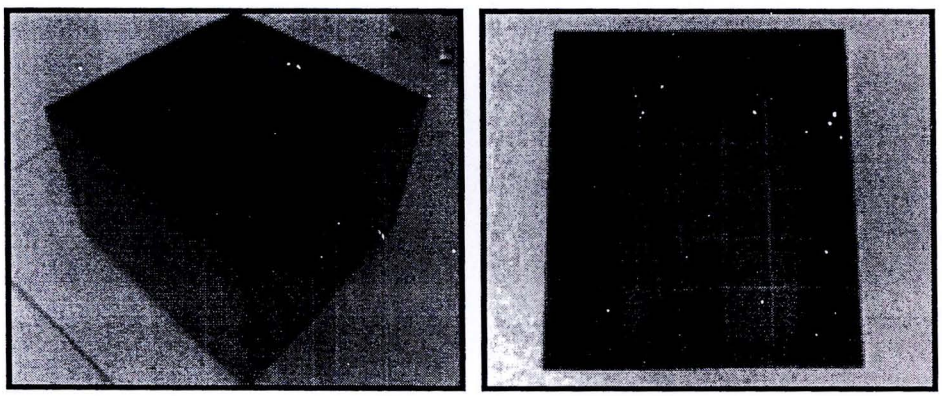

Figure 6 Open-field apparatus for testing locomotor activity.

5. Western blot

5.1 Brain sample preparation

The animals were killed by rapid cervical dislocation after the last behavioral observation. The whole brains removed from their skull were frozen and stored at -70°C until used. The brain was homogenized in 1 ml (10 fold of brain volume) of lyses buffer (final concentration; 0.01 M Tris-HCl, 0.05 M ethylenediaminetetraacetic acid (EDTA), 5% sodium dodecyl sulfate (SDS), 7.5% sodium deocxycholate (DOC), 1 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 0.88% Triton X-100, 75 mM NaCl, 1.25 mM NaF, pH 7.4) at 4 °C containing 1% cocktail protease inhibitor (Sigma-Aldrich, Inc., St. Louis, MO, USA) in a 10 ml glass Wheaton tissue grinder and were grinded for 4 strokes. The tissue grinder was rinsed out with distilled water. A sample (approximately 1000 µl or above) was kept as "whole tissue homogenate". It was centrifuged for 60 minutes with 15,000 g at 4 °C and supernatant was collected. The supernatant was assayed for protein and used for western blotting for receptor's protein expression assay. The MicroBCA™ protein assay kit (Thermo Fisher Scientific, Inc., IL, USA) was used for determination of protein concentration.

5.2 Gel-electrophoresis and Immunobloting

Changes in expression of nicotinic and muscarinic receptors were determined by western blot using antibodies specified for alpha7 containing nicotinic acetylcholine receptors (nAChRα7, H-302, Santa Cruz); and M5 muscarinic acetylcholine receptor (mAChRM5, H-197, Santa Cruz).

Equal amounts of protein (75 µg) in each sample were separated by SDS–PAGE (10%, w/w, gel) at 25 mA and electrotransferred to polyvinyllidene fluoride (PVDF) membrane overnight at 35 Volt. The PVDF membrane was blocked with 5% non-fat dry milk in washing buffer for 60 minutes. After blocking, the membrane was washed and then incubated with primary antibody (1:300 dilutions) in 2.5% non-fat dry milk in washing buffer for overnight. After three 10 minutes washes in washing buffer, the PVDF membrane was then incubated in the secondary antibody (1:10000 dilutions) in 2.5 % non-fat dry milk in washing buffer for 60 minutes. After three 10 minutes washes in washing buffer, proteins were then visualized using the enhanced chemiluminescence (ECL) detection kit (Pierce, IL, and USA) and exposed

to CL-Xposure™ film (clear blue x-ray film) in Hypercassette® for 1 to 5 minutes. Band intensity were measured using a Quantity One® software package version 4.6.5 (Bio-Rad, CA, USA). The expression levels were calculated from the specific band intensity.

6. Receptor displacement assay

6.1 Brain sample preparation

Membrane homogenates were prepared by homogenizing whole mice brain with 10 volume of 50 mM Na:K phosphate buffer, pH 7.4 on ice in a 10 ml glass Potter-Elvehjem Tissue Grinders. The homogenates were centrifuged at 15000 g for 10 minutes at 4°C. The membrane pellets were washed twice in 10 volume of ice-cold Na:K phosphate buffer and were stored at -70°C until use.

6.2 Ligand binding to brain tissue homogenate

Receptor displacement assay was performed using 96-well plate. Nicotinic receptor displacement assay was made using 35 µl aliquots of VE that obtained from dilution series (0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, and 10.0 mg/ml in distilled water). VE from the dilution series were added to 230 µl of homogenized membranes equivalent to 300 mg protein/ml and then suspended in 50 mM Tris–HCl containing 8 mM $CaCl_2$, pH 7.4 and muscarinic receptor displacement assays was performed using a same protocol, except 20 mM HEPES containing 10 mM $MgCl_2$ and 100 mM NaCl, pH 7.4 was used as buffer. After 30 minutes of pre-incubation at 25°C, 35 µl of [^{3}H]-nicotine was added to give a final [^{3}H]-nicotine concentration of 4 nM for nicotinic receptor displacement assay and 35 µl of [^{3}H]-scopolamine was added to give a final [^{3}H]-scopolamine concentration of 1 nM for muscarinic receptor displacement assay. The homogenates were incubated for 60 minutes at 25°C and then were harvested with ice-cold buffer by the Filtermate harvester (PerkinElmer, Inc., MA, USA). Radioactivity was measured using the TopCountNXT™ scintillation counter (PerkinElmer, Inc., MA, USA). Non-specific binding were defined as residual binding in the presence of 10 µM carbachol for [^{3}H]-nicotine binding and 1 µM atropine for [^{3}H]-scopolamine binding. Displacement curves were conducted from fMol bound/mg protein against logVE concentration [73].

7. Receptor autoradiographic studies

7.1 Tissue preparation

Ten micrometers of brain sections were obtained using Leica CM1850 cryostat (Leica Microsystems Inc., IL, USA). In mouse stereotaxy, bregma is a reference point, classically defined as the endpoint of the olfactory bulb (brain) or the midpoint of the curve of the skull (Figure 7) [74, 75]. Hippocampus was collected starting at bregma 1.94 mm and ending at 0.74 mm, ventral tegmental area was collected starting at bregma -2.30 mm and ending at -3.08 mm, and nucleus accumbens was collected starting at bregma -2.92 mm and ending at -3.88 mm [74]. The sections were mounted on silane coated slides and stored at −70°C until used.

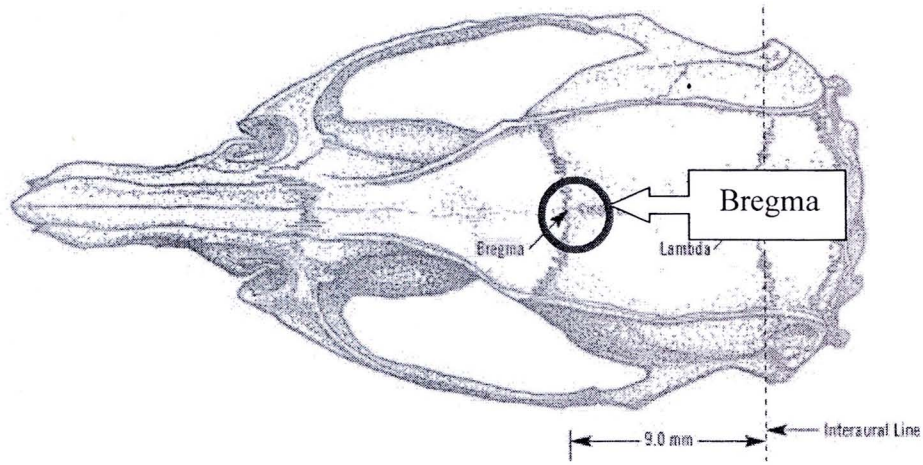

Figure 7 Rodent's skull diagram [74].

7.2 Autoradiographic procedures

For the autoradiographic localization of nicotinic receptors, the sections were pre-incubated at room temperature for 15 minutes in 50 mM Tris-HCl with 120 mM NaCl, 5 mM KCl, 2.5 $CaCl_2$, and 2 mM $MgCl_2$. Sections were then incubated in 4 nM [^{3}H]-nicotine (specific activity = 75 Ci/mmol) in the Tris-HCl buffer with 120 mM NaCl, 5 mM KCl, 2.5 $CaCl_2$, and 2 mM $MgCl_2$, for 240 minutes at room temperature followed by incubation in the Tris-HCl buffer for 2 minutes. Non-specific binding was assessed in the presence of 10 μM unlabeled nicotine during incubation.

For the autoradiographic localization of muscarinic receptors, sections were pre-incubated at room temperature for 15 minutes in 10 mM KH_2PO_4 with 10 mM Na_2HPO_4, for 15 minutes. Sections were then incubated in 1 nM [^{3}H]-scopolamine (specific activity = 76 Ci/mmol) in the phosphate buffer for 60 minutes at room temperature followed by incubation in phosphate buffer for 2 minutes. Non-specific binding was determined as that persisting in the presence of 10 μM atropine during incubation.

After the incubation period, sections were dipped twice in the same buffer, rinsed in distilled water and dried under a warm air stream. Autoradiographs were generated by exposing the labeled tissue sections to tritium-sensitive films for 4 weeks in nicotinic receptor assay and for 2 weeks in muscarinic receptor assay.

Autoradiographic [^{3}H] micro-scales were used as the standard for estimate tissue section equivalent. Receptor binding were quantified using Quantity One® software version 4.6.5 (BIO-RAD Laboratories, Inc., CA, USA)

Statistical analysis

Data from the anxiety-like behavior, locomotors activity, total abstinence signs, autoradiographic, immunoblotting, and receptor displacement assays were evaluated using the one-way analysis of variance (ANOVA) followed, when appropriate, by post hoc comparison using Tukey test. Significant levels were considered at $P < 0.05$.