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Unconventional Raw Natural Sustainable Sources for Obtaining Pharmacological Principles Potentially Active on CNS Through Catalytic, Ecologically Clean, Processes

Yanier Nuñez Figueredo¹, Juan E. Tacoronte Morales²,
Alicia Lagarto Parra¹, Olinka Tiomno Tiomnova²,
Jorge Tobella Sabater³ and Jorge Leyva Simeon²

¹*Center for Research and Development of Pharmaceuticals,
CIDEM, Cerro, Ciudad Habana,*

²*Center for Engineering and Chemical Researches, CIQ, Cerro, Ciudad Habana*

³*Pharmaceutical Laboratories MEDSOL, La Lisa, Ciudad Habana,
Cuba*

1. Introduction

Natural products have been the most successful source of drugs ever (Tulp & Bohlin, 2005). Historically, the most important natural sources have been plants. Research progressed along two mayor lines: ethnopharmacology and toxicology. These strategies have produced many valuable drugs and are likely to continue to produce hit-lead compounds. However, actually exist numerous unconventional natural sources, ecologically sustainable, of potentially medicinal compounds without research.

The development of pharmaceutical and fine chemistry in Cuba and the synthetic or natural product-oriented generation of new pharmacological and molecular entities, in the II decade of XXI century, it's sustained in several basic conceptual and methodological principles:

- Structure (including isosteric perception) of compounds generates and define properties, which in their order, determinate application and functionality in the related chemical-pharmacological space (SPAF)
- Sustainability – Scalability (scaling-up facilities) –Applicability in real time (SSA-r_t)
- Maximum of atomic efficiency (*click* and *green* chemistry); maximum of analytical efficiency and maximum of environmental efficiency (MAE³)
- Chemical bioprospecting of the Cuban biodiversity oriented to discovery of new *ecological* molecular fragments-templates with interesting pharmacological properties for their application in therapeutical treatments of several pathologies of CNS including neuroprotection, neuroregeneration after stroke and ischemia.

- Integration of *in silico* screen to natural products or their mixtures with minimal complexity

Taking this into account, in Cuba (2008-2011) it has been attempted, starting from natural products of the forest industry and raw renewable materials ecologically sustainable, such as rosin, colophony and resinic acids isolated from endemic botanic species belonging to gen. *Pinus* (Pinaceae) the development and application of new heterogeneous catalytic procedures, optimization of design and synthesis of new pharmacological agents structurally based on sodium resinate and dehydroabietic acid (DHAA), with a great added value, as therapeutics for medical treatment of pathologies of CNS, including GABA agonist-antagonists, cannabinoid analogues and sedative molecular systems.

Cuban resin acids, an ecological sustainable natural product, derived from Cuban forestry industry, have been shown to have broad and highly active biological properties, potent microbiocidal and fungicidal actions and potential neuroprotective effects on central nervous system (CNS). In this communication, we studied this novel ecologically sustainable source of potentially therapeutic compounds using, as starting raw material, resins from endemic Cuban *Pinus* specie and show their effect on central nervous system in rodents.

2. Materials and methods

2.1 Chemicals and drugs

2.1.1 Oleoresin, colophony and starting resin acids

The Cuban oleoresin, was milked and collected by incision of the bark from mature trees (*Pinus caribbaea* 7-13 years) planted in Viñales Forestry Station, 168 Km West of Havana, serpentine soil) located in the Western zone of the Cuban archipelago. It was submitted to distillation as reported previously (Franich & Gadgil, 1983). The main components, a mixture of colophony (resin acids), turpentine oils and neutral fraction were separated. The acid fraction was submitted to identification of abietane acids (mixture of abietic, dehydroabietic, levopimaric & pimaric acid) and directly used in pharmacological tests.

For the preparation of extracts, approximately 500 mg of colophony were grinding in an agate mortar into 2-mm pieces, extracted with 4 ml methanol, filtered through glass wool, and the extracts stored at -10°C . Extracts were filtered through 0.45- μm Teflon syringe filters prior to HPLC and used in the next step for obtaining the sodium salt.

The sodium salt (sodium resinate-SR) was prepared as reported in: CU/2006-0144 patent. Crystallized from a mixture of water: ethanol (8: 2 v/v)

2.2 Chromatography conditions

Analytical HPLC-PDA (Kanuer Smart Line-2005): Column: LichroCART 250 x 0.44 cm RP-18, 5 μm particle size (Lichropher 100); mobile phase: acetonitrile-water in gradient conditions (40% to 100%) during 45 min, held at 100% for further 2 min; temperature: 25 $^{\circ}\text{C}$; flow rate: 1mL/min; sample injection 10 μL ; detection: 200 nm to 505 nm. Data were analyzed with ChromGate 3.1 (Germany) for LC 3D software.

2.3 Structural elucidation

The structural elucidation was based on FTIR spectroscopy (spectrophotometer FT-IR Jasco, FT/IR-460 Plus, Japan, in a range of 280-7200 cm^{-1} with a sensibility of 0,1 cm^{-1}) and NMR spectrometry (^1H y ^{13}C) at room temperature, using a spectrometer Bruker AC-250 MHz, at 28 $^{\circ}\text{C}$ in DMSO-d_6 , using as internal reference TMS, given all the signals in ppm (δ).

Diazepam (DZP, Quimefa, Cuba), pentylenetetrazole (PTZ, Sigma, USA, CAS), picrotoxin (PTX, Sigma, USA, CAS), haloperidol (Esteve S.A, España), amphetamine-sulphate (Sigma, USA, CAS), were used in this study. Solvents were analytical grade and were purified by distillation before used. All drugs and their solutions were prepared immediately before use.

2.4 Synthesis of dehydroabietic acid (DHAA)

2.4.1 Catalytic disproportion of colophony using piritic ash as catalyst (0,5 % (m/m) at 230 $^{\circ}\text{C}$)

500 grams of previously hydrothermally treated colophony is heated during 30 min (130 $^{\circ}\text{C}$) in a glass pyrex reactor (750 mL) equipped with thermometer and stirrer. 1,0 gram of pyritic ash is added and the reaction temperature increased to 230 $^{\circ}\text{C}$. The reaction mixture is maintained at this temperature during 3 h under intense stirring (1500 rpm). (The residual concentration of abietic acid is 0,5-0,8 %). 100 grams of disproportionated colophony in 100 mL of alcohol is filtered through $\text{SiO}_2\text{-Al}_2\text{O}_3$. The solution is heated to 70 $^{\circ}\text{C}$ and then added 18 grams of 2-aminoethanol, and 250 mL of hot water (60-90 $^{\circ}\text{C}$). The resulting solution is kept at 70 $^{\circ}\text{C}$ during 10 min., under gentle stirring, then the reaction mixture is extracted with iso-octane, toluene or a mixture of heptane/ciclohexanone 7/3 v/v (3v x 75 mL). The selective crystallization of the quaternary ammonium salt dehydroabietic acid-2-aminoethanol starts at 50 $^{\circ}\text{C}$. The solution is cooled to 4 $^{\circ}\text{C}$, collecting crystals which are soluble in cool 50 % ethanol (250 mL) Yield: 51,0 grams with 89,5 % of purity related to dehydroabietic acid (98,5 % overall purity). The obtained salt is dissolved 160 mL of hot ethanol and acidified with aqueous HCl (12 %, pH 4-5) and allowed to stand during 8h at room temperature. The pure dehydroabietic acid is collected, washed, re-crystallized from a mixture of 75 % ethanol/water v/v. and dried 3 h. Yield 39,3 % (overall 98,2 %)

2.5 Animals

2.5.1 Pharmacological studies

Male albino mice (Swiss, 18-22 g) in anticonvulsant activity, elevated plus-maze, amphetamine and thiopental-induced sleep, open field activity and aggressive behaviour test and male rats (Wistar, 150-200 g) in amphetamine-induced behavioural stereotypy test were used.

2.5.1.1 Acute toxicity study

Six female rats (Wistar, 150-200 g) were used for evaluating the acute toxicity of test compounds.

All animals (Laboratory of Biological Control. CIDEM, Havana, Cuba) were housed in groups of five under standard laboratory conditions of temperature, humidity and lighting (12:12-h light/dark). Animals had free access to food and water, except during experiment.

They were deprived of food but not water 6 h before the drug administration and each group consisted of ten animals. All experiments were carried out between 8:00 am and 11:00 am in accordance with the Institutional Animal Ethical Committee approved the study and animal care was in conformity with Canadian Council for Animal Care guidelines

SR was administrated in three doses levels (100, 200 y 400 mg/Kg) in all experiment except the elevated plus-maze behaviour test (50, 100 y 150 mg/Kg). The volume of injection in mouse was 0.4 ml/20 g and in rat was 1 ml/100 g. The SR was dissolved in distilled water and administered orally.

2.5.2 Pharmacological and toxicity evaluation

2.5.2.1 Open field activity

Thirty minutes after the administration of vehicle or test compound a mouse was placed in the centre of a round open field of 30 cm diameter and 25 cm high and the open field activity were measured during 6 minutes recording how many times the animal stay in the centre of cage and the number of rising (Sukma et al., 2002; Tyler, 1982).

2.5.2.2 Aggressive behavior

A group of animal was isolated in individually cage and other group remained grouped during six week. The aggressive behaviors were evaluated through an intruder mouse into the isolated mice's home cage and were recorder the aggressive activity (biting attacks and wrestling) in isolated mice was measure as total fighting time during a 20 min period (Tyler, 1982).

2.5.2.3 Thiopental-induced sleep

Animals were divided into three groups: control (distilled water), diazepam (1 mg/kg)-treated and SR-treated groups. Thiopental sodium (30 mg/kg) was injected intraperitoneally 30 min after administration of vehicle or test compound. An animal was placed on its back on a warmed (35 °C) pad. The number of sleeping animals and the duration of loss of righting reflex were recorded. The duration from loss of righting reflex until a mouse regained its righting reflex was measured (Carlini et al., 1986).

2.5.2.4 Drug-induced convulsion

Mice were divided in groups of ten each. The animals were pre-treated with SR 30 min before the administration of PTZ (85 mg/kg, s.c.) or PTX (10 mg/kg, s.c.). The anticonvulsive effect was assessed by measuring the numbers of convulsing mice and deaths, and the latency of the appearance of the first episode of clonic seizure. The cut off time was set as 30 min. after the convulsant administration (Costa & Greengard, 1975; Fischer & Vander, 1998).

2.5.2.5 Elevated plus-maze behavior

The elevated plus-maze consisted of two closed arms (30_/5_/15 cm) and two open arms (30_/5 cm) emanating from a common central platform (5_/5 cm). The two pairs of identical arms were opposite each other. The entire apparatus was elevated to a height of 54 cm above floor level. Thirty minutes after test compound administration, the mouse was placed

at the centre of the maze with its head facing an open arm and allowed to explore the maze for 5 min. Entry into an arm was defined as placement of all four paws into an arm and were recorded: number of entries into each type of arm, the percentage of time spent and the percentage of arm entries in open arms (Sukma et al., 2002).

2.5.2.6 Amphetamine-induced behavioral stereotypy

Amphetamine (1.5 mg/Kg) was injected subcutaneously 30 min after administration of vehicle or test compound in rats and the animals were collocated in individually cage to recorder the behavioral stereotypy each 5 min during 1 h. (Kuczenski et al., 1999).

2.5.2.7 Amphetamine -induced sleep in mice

Mice were divided in four groups of ten each. The animals were pre-treated with SR 30 min before the administration of amphetamine 5 mg/Kg (p sc). An animal was placed on its back on a warmed (35 °C) pad and the number of sleeping animals and the duration of loss of righting reflex were recorded.

2.5.2.8 Acute oral toxicity study

A single dose of SR (2000 mg/kg) or distilled water was administered orally (10 mL/Kg) in equal number female (n=3) animals; and rats were returned to an *ad libitum* diet immediately after dosing. All animals were monitored continuously for 12 h after dosing for signs of toxicosis and daily for changes additional behavioural or clinical signs. The animal weights were recorded weekly. Rats were euthanized on day 14 by ether inhalation, and selected organs removed and examined macroscopically for toxicant-induced changes (OECD, 2001).

2.5.2.9 Statistics

Drug effects were assessed by single factor analysis of variance followed by the Student-/Newman-/Keuls post-hoc test. The level of significance was set at $p < 0.05$.

3. Results and discussion

The Cuban pine oleoresin, was milked and collected by incision of the bark from mature trees *Pinus caribbaea*. After distillation the main components and mixture of colophony (resinic acids), turpentine oils and neutral fraction were separated and hydrothermally treated to minimize the amount of fatty lineal and branched acids. Colophony was analyzed, treated with stecheometrical amount of NaOH, to obtain the desired sodium resinate and submitted directly, after crystallization from a mixture water: ethanol (30:70 v/v), to neuropharmacological evaluations.

The oleoresin collected from *Pinus caribbaea* is hydrothermally treated and purified through redox-acid/base protocols described in (Cuban patents CU 20060144 & CU20060252), generating a practically pure mixture of rosin-colophony as a mixture of resinic acids (RA, FTIR- cm^{-1} : 3426(γ O-H), 2931($\text{C}_{\text{sp}3}$ -H y $\text{C}_{\text{sp}2}$ -H), 2869 (ν^s CH_2) y 2929 (ν^{as} CH_2), 1694($\gamma\text{C}=\text{O}$), 1385-1366(δ_s - CH_3), 1450(δ_{as} - CH_3), 1150-1180 isopropyl system, zone 950-970 olefinic fragments, 830-770 trisubstituted olefins; NMR, δ ,ppm: 6 zones observed: 0.5-0,8 methyl groups, 1.0-1.2 methyl groups, 1.3-2.0 methylenic groups, 5.0-6.0 olefinic zone exo- and endocyclic bonds, 6.8-7.3 aromatic protons, 11.9-12.5 COOH) that was used directly in the evaluation of its neuropharmacological profile. This mixture (50-52 % of abietic acid),

without further purification, was used for obtaining dehydroabietic acid (DHAA) by heterogeneous catalytic disproportionation-aromatization treatment of colophony with pyritic ash ($\text{Fe}_2\text{O}_3/\text{FeS}/\text{Ba}^{2+}/\text{SiO}_2$, 230°C, stirring, air, 95%) and the selective precipitation of the 2-aminoethanol salt of DHAA (molar ratio 1:1, 50°C, 89 %) in aqueous ethanol solution. The pure DHAA was obtained after acidification (pH 4-5; 98%, TLC: Silicagel G₆₀-254, eluent: n-hexane/ethyl acetate 7:3 v/v, 2 drops of isopropanol; chromophoric agent vainilline/ H_2SO_4 , mp. 171.5-172.3°C and applied column chromatography; NMR, ppm, δ ^1H - ^{13}C selected signals: 9,78-OH/184,32-COOH; 7,15 C-11H/C-11 124,90; 6,95 C-12H/C-12 124,20; 6,88 C-14H/C-14 127,0).

The most widely protocol used for resin acid analysis and their derivatives is gas chromatography (GC) of the methyl esters (Zinkel & Engler, 1977) with detection by flame ionization (FID) or mass spectrometry (MS). However, this method has disadvantages for us, including instability of the derivatized samples (Latorre et al., 2003), hazards of methylating reagents (potentially explosive and carcinogenic), and tedious work-up of raw biological material required.

Taking this in consideration, in our laboratory, have been developed a simple analytical methodology for resin acid analysis by high-performance liquid chromatography (HPLC) in gradient conditions. We report a simple protocol for the analysis of abietanes derivatives by reversed-phase HPLC with several advantages including: (1) no sample derivatization is required; (2) extraction and chromatographic conditions are mild, (3) all components of the HPLC mobile (acetonitrile and water) phase are volatile and therefore recovery of compounds from fractionated sample is simplified. These benefits are particularly advantageous in biological studies that require rapid analysis of abietane acid mixtures and screenings for neuroprotective bioactivity.

The results are shown in Fig. 1

Fig. 1. Abietanes (resin acids present in the Cuban colophony) HPLC analysis of methanol extract obtained from distilled oleoresin. 1= Levopimaric, 2= Palustric, 3= Abietic, 4= Dehydroabietic

The fundamentals (conceptual and methodological) for the analytical technique described here rely on both the optimal combination of wavelengths for detection and the chromatographic resolution of the peaks. Abietanes have distinctive spectra that we used here, together with chromatographic separation, to distinguish and quantify the resin acids by HPLC. The spectra (220-540 nm) of the individual chromatographically separated components (Fig.1), show $\lambda_{\max}$ values of 255 nm (levopimaric), 251 nm (palustric), 266 nm (abietic), and 269, 278 nm (dehydroabietic). The information related is shown in Fig. 2.

Fig. 2. Spectral chromatogram of analyzed mixture of resin acids present in Cuban colophony

The developed HPLC analysis revealed the main components of the Cuban colophony, a starting raw material for preparing sodium resinate (Fig. 3).

Fig. 3. Main resin acids present in the Cuban colophony. (abietic acid 40 %; dehydroabietic acid 22 %; palustric acid 18 %; levopimaric acid 18-20 %).

The colophony was administered in 3 dosis levels (100, 200 y 400 mg/Kg) in all experiments, except in the case of bioassay of labyrinth in cross (50, 100 y 150 mg/Kg). The DHAA dosis levels were 50, 100 and 200 mg/Kg. The injection volume of administration in mice was 0.4 ml/20 g and 1mL/100 g in rats. The colophony (sodium salt-SR) was dissolved in distilled water and administered orally.

3.1 Open field activity

SR (100, 200 and 400 mg/kg, po.) reduced locomotor activity and rearing in a dose-dependent manner during the observation period. The observations are given in Table 1. Doses of 400 mg/Kg of SR showed similar behaviour to diazepam (DZP) 1 mg/Kg (standard anxiolytic drugs).

The present study demonstrated that SR prepared from natural and pre-treated (hydrothermally and by acid-basic reaction) resin extracted from Cuban *Pinus* reduced spontaneous locomotor activity in mice. Usually the rodents show an exploratory behaviour when they are collocated in a novel place. However, if the animals are pre-treated with depressant central nervous system drugs, the locomotor activity is decreased. This result is typical for sedative drugs.

Tested groups	mean±S.E.M
Distilled water	24,5 ± 4,20a
SR 100 mg/Kg.	20,2 ± 3,38 b
SR 200 mg/Kg.	19,6 ± 2,22 b
SR 400 mg/Kg.	12,9 ± 4,48 c
DZP 1 mg/Kg.	10,5 ± 3,39 c

Table 1. Effects of SR (100, 200 and 400 mg/kg, po.) on spontaneous locomotor activity. Groups with unequal letters differ to each other for p< 0.05.

3.2 Aggressive behavior

Social isolation induces aggressive behavior in several strains of mice. The isolation-induced aggression is proposed to be useful as an animal model for assessing inhibitory activity on central nervous system. Different neurotransmitters such as serotonin, noradrenaline, dopamine and gamma-aminobutyric acid (GABA) are considered to be involved in mediating aggressive behaviour; there are conflicting results on brain neurotransmitter metabolism (Matsuda et al., 2001; Sakaue et al., 2001). Table 2. shows the effects of SR on aggressive behaviour in isolated mice. Test compound reduces an aggressive behaviour in a dose-dependent manner. A similar result to open field test in between 400 mg/Kg of SR and diazepam 1 mg/Kg doses was obtained. This behaviour was reported by Valzelli in 1973 as a classic pattern for central nervous system depressor (Valzelli, 1973). Our results show an anti-aggressive behaviour in orally SR-treated mice. This result can be mediated by inhibitory effects on brain biogenic amines action or excitatory neurotransmitter release and suggests the inhibitory effect of SR on the central nervous system.

Tested groups	mean±S.E.M
Distilled water	24.90 ± 4.15 a
SR 100 mg/Kg.	17.60 ± 3.38 b
SR 200 mg/Kg.	16.75 ± 2.94 b
SR 400 mg/Kg.	12.90 ± 4.48 c
DZP 1 mg/Kg.	10.50 ± 3.39 c

Table 2. Effects of SR (100, 200 and 400 mg/kg, po.) on aggressive behaviour (biting attacks and wrestling) in isolated mice Groups with unequal letters differ to each other for p<0.05.

3.3 Thiopental-induced sleep

SR as well as diazepam, a standard reference drug, increased the number of sleeping animals and prolonged the thiopental-induced sleeping time in mice.

All SR doses increase the number of sleeping animals (Table 3) compared with the control, doses of 200 and 400 mg/Kg caused sleep in all animals.

SR (400 mg/Kg) and diazepam (1 mg/Kg) prolonged thiopental induced sleep in the similar manner (Table 4).

Tested groups	Percentage of sleeping animal
Distilled water	12.50
SR 100 mg/Kg.	56.25
SR 200 mg/Kg.	100
SR 400 mg/Kg.	100
DZP 1 mg/Kg.	100

Table 3. Effect of SR on percentage of sleeping animal.

Tested groups	mean±S.E.M
Distilled water	2,25 ± 6.16 a
SR 100 mg/Kg.	8,25 ± 8.65 b
SR 200 mg/Kg.	33,75 ± 8.83 c
SR 400 mg/Kg.	40,00 ± 0 d
DZP 1 mg/Kg.	40,00 ± 0 d

Table 4. Effect of SR on sleeping time (min). Groups with unequal letters differ to each other for p<0.05.

3.4 Drug-induced convulsion

Numerous excitatory drugs such as PTZ and PTX, can induce convulsion *via* GABA receptor antagonism, due to, the anxiolytic-like drugs (ex. diazepam) might be inhibit the drug-induced convulsion via inhibition of GABA-ergic inter-neurons.

To further investigate the inhibitory effect of SR on the central nervous system, drugs that can excite or block excitation in the central nervous system were used. Diazepam (4 mg/kg, po.) was highly effective in delaying the occurrence of clonic convulsion (Table 5 and 6) even in protecting animals against convulsion induced by PTX and PTZ. However the different doses of SR tested were unable to protect animals against convulsion and death, and unable to increase the latency of the clonic convulsion induced by pro-convulsivant drugs. Our findings show an ineffective action of SR to avoid the PTZ and PTX-induced convulsion. These results suggest that the sedative effects of test compound might not be mediated via GABA or glycine systems.

Tested groups	Latency of first convulsion	Latency of first clonic convulsion	Time of death	Percentage of animals with clonic convulsion	Percentage of death
PTX 5 mg/Kg + Distilled water	12.0 ± 1.94a	20.66 ± 4.55a	22.66 ± 5.03a	80	30
PTX 5 mg/Kg + SR 100 mg/Kg.	12.0 ± 6.00a	17.0 ± 4.36a	23.0 ± 8.48a	70	20
PTX 5 mg/Kg + SR 200 mg/Kg.	15.90 ± 8.60a	20.2 ± 4.76a	26.0 ± 1.41a	80	20
PTX 5 mg/Kg + SR 400 mg/Kg.	16.8 ± 6.37a	21.62 ± 5.70a	24.0 ± 2.91a	80	20
PTX 5 mg/Kg + DZP 4 mg/Kg	23.0 ± 3.21b	26.0 ± 3.80b	28.1 ± 2.12b	20	0

Table 5. Effect of SR on clonic convulsion induced by PTX. Groups with unequal letters differ to each other for p<0.05.

Tested groups	Latency of first convulsion	Latency of first clonic convulsion	Time of death	Percentage of animals with clonic convulsion	Percentage of death
PTZ 85 mg/Kg + Distilled water	7.30 ± 1.66 a	11.66 ± 3.07 a	14.80 ± 3.83 a	60	50
PTZ 85 mg/Kg + SR 100 mg/Kg	6.00 ± 2.45 a	11.50 ± 1.52 a	13.17 ± 3.97 a	60	60
PTZ 85 mg/Kg + SR 200 mg/Kg	7.27 ± 1.35 a	13.16 ± 2.23 a	13.0 ± 3.16 a	60	40
PTZ 85 mg/Kg + SR 400 mg/Kg	7.90 ± 2.28 a	11.50 ± 3.45 a	13.33 ± 1.86 a	60	40
PTZ 85 mg/Kg + DZP4 mg/Kg	25.0 ± 2.18 b	27.1 ± 3.26 b	29.0 ± 1.18 b	20	0

Table 6. Effect of SR on clonic convulsion induced by PTZ. Groups with unequal letters differ to each other for p<0.05.

3.5 Elevated plus-maze behavior

Rodents usually avoid open arms and prefer enclosed arms in an elevated plus-maze. Time spent in open arms and numbers of entries into open arms are indexes of neophobic anxiety in animals. Standard anxiolytic drugs such as diazepam increase open-arm exploration, as reflected by increases in the time spent and the number of entries into the open arms (Pellow, 1985).

Diazepam exhibited the conventional profile of anxiolytics in the elevated plus-maze test; it increased the percentage of open arm entries and time spent in open arms (Table 7). However, SR did not significantly modify the percentage of either time spent or arm entries in open arms at any of the doses tested.

Tested groups	Time spent in open arms	Time spent in close arms	Open arm entries (%)	Open close entries (%)	Number of open arm entries (counts)	Number of closed arm entries (counts)
Distilled water	8.2 ± 8.02 a	198.9 ± 44.70 a	14.81 ± 12.45 a	85.18 ± 12.45 a	1.80± 1.62 a	8.9± 2.33 a
SR 50 mg/Kg	9.12 ± 7.07 a	112.56 ± 30.25 a	16.23 ± 14.36 a	87.26 ± 10.25 a	1.93± 0.98 a	8.03± 2.15 a
SR 100 mg/Kg	9,6 ± 14,35 a	85.4 ± 42,77 a	18,68 ± 23,70 a	81,31 ± 23,70 a	1.50± 2.01 a	7.6± 3.60 a
SR 150 mg/Kg	10.08 ± 8.23 a	93.15 ± 29.15 a	17.59 ± 9.23 a	83.26 ± 15.15 a	1.75± 1.29 a	7.98± 2.23 a
DZP 0.5 mg/Kg	55.23 ± 5.56 b	70.18 ± 7.83 b	65,17 ± 12.18 b	34,83 ± 6.65 b	19.05± 2.15 b	10.18± 1.98 a

Table 7. Effect of SR on the elevated plus-maze test in mice (see Table 6). Groups with unequal letters differ to each other for p<0.05.

3.6 Amphetamine-induced behavioral stereotypy

Psycho stimulants (such as, amphetamine a simpatic-mimetic amine) administration in rats increase dopamine levels in different brain zones, induce stereotyped behaviours, characterized by repetitive sniffing, biting, grooming, and head movements. The dopamine receptor antagonist or sedative drugs can be reducing its behaviour (Ralph et al. 2001). Table 8 shows behavioral stereotypy after subcutaneously administration of amphetamine, 1.5 mg/Kg (p.sc). Rats treated with SR reduced its behaviour in a dose-dependent manner compared with the water-treated group and amphetamine. The observed results reveal that colophony decreases the stimulant effect either for an antagonism of dopaminergic transmission, inhibition of dopamine releasing, blocking of its post-synaptic receptor or by an activation of some inhibitory transmission with the decrease of excitation caused by the dosis of employed amphetamine. The data suggest that the colophony has not a characteristic profile of active antidepressants. It was confirmed by the evaluation of amphetamine (5 mg/Kg)-induced sleeping and its extension in time.

Tested groups	mean±S.E.M
Distilled water	10.45 ± 3.70a
SR 100 mg/Kg. + amphetamine 1,5 mg/Kg.	22,71 ± 4.90b
SR 200 mg/Kg. + amphetamine 1,5 mg/Kg.	23,92 ± 5,75b
SR 400 mg/Kg. + amphetamine 1,5 mg/Kg.	20,70 ± 5,85b
Distilled water + amphetamine 1,5 mg/Kg.	31,64 ± 5,98c
Haloperidol 5 mg/Kg. + amphetamine 1,5 mg/Kg.	12,28 ± 2,56a

Table 8. Effect of SR on behavioural stereotypy induced by amphetamine. Groups with unequal letters differ to each other for p<0.05.

3.7 Amphetamine -induced sleep in mice

Mice treated with different doses of SR exhibited similar behaviour against amphetamine - induced sleep in mice compare with water-treated group (Table 9).

Tested groups	Media ± D.S
Distilled water + amphetamine 5 mg/Kg	189.90 ± 9.65
SR 100 mg/Kg. + amphetamine 5 mg/Kg	190.00 ± 11.51
SR 200 mg/Kg. + amphetamine 5 mg/Kg	195.86 ± 5.05
SR 400 mg/Kg. + amphetamine 5 mg/Kg	190.22 ± 7.07

Table 9. Effect of SR on sleep induced by amphetamine in mice.

Although SR showed a sedative effect in evaluated tests (thiopental-induced sleep, open field activity, aggressive behaviours and amphetamine-induced behavioral stereotypy). The interaction of SR with convulsant drugs, confirms that the sedative effect of test compound might not be related via the GABA or glycine systems.

In this context, recently our group found that abietic and dehydroabietic acids might be responsible of the sedative effects of the SR (Unpublished result).

3.8 Acute oral toxicity study

The death of any animal was not observed during the study. The major adverse effects were related with CNS depression (motor impairment and sedation), but these symptoms

disappeared within 4 h. With this exception, no outward behavioral abnormalities were noted during the 2-week post-treatment period. The body weight gains were observed in the similar manner in both groups (Figure 4). Macroscopic alterations were not observed in selected organs and tissues removed (stomach, liver, kidney, brain, spleen and lungs). The administration of SR didn't cause significant toxic symptoms for what classifies in 5 category of the GHS or not classified (mortality> 2000 mg/kg).

Fig. 4. Effect of SR 2000 mg/Kg on acute oral toxicity test.

Taking into consideration the structural similarity, *grosso modo*, (molecular modeling are underway and are unpublished results yet) between secondary metabolites, DHAA, related derivatives and cannabinoids, the observed pharmacological effects could be attribute to a potential antagonism with glutamate receptors and/or inhibition of noradrenalin-dopamine releasing. The acute toxicity study revealed that no toxic effects were observed, and any symptom (increase of cleaning-up behavior, exploration, decreasing of frequency of movement, etc) disappeared after 4 hrs. In the autopsy were not detected any macroscopic or anatomic effect on organs.

In the case of dehydroabietic acid (DHAA) generated by disproportionation-aromatization of resinic acids the results reveal a dosis depending effect on decreasing of

exploratory activity, typical for sedative pharmaceutical compositions. Its co-administration (50 mg/Kg) with thiopental, at experimental dose, increased the number of sleeping animals (65%), a typical depressing action on CNS. The administration of DHAA, at all doses evaluated, didn't protect animals for PTZ induced convulsions. The experimental data obtained in the Labyrinth in cross model didn't show any relevant results. In the Amphetamine-induced stereotypia model DHAA decreases the stimulant action produced by the subcutaneous administration of 1,5 mg / Kg of amphetamine, in the hypothetically pathways described above. The acute toxicity ($DL_{50} > 5000$ mg/Kg) study showed that DHAA had not any toxic effects at dose concentrations used and could be classified in the GSH in the 5 category.

It is noteworthy that synthetic DHAA has a sedative action on CNS and could be employed as starting raw material for designing of new molecular and pharmacological entities potentially useful in the development of formulation for therapy of CNS pathologies where a sedative effect is needed.

Topological studies for determining any QSAR correlation between colophony and derivatives and cannabinoids are underway.

4. Conclusion

The oleoresin, a raw material isolated from Cuban Pinacea (gen. *Pinus*, *Pinus caribbaea*), constitutes a sustainable resource for developing potentially useful pro-drugs and pharmacologically active substances as resinic acids, sodium resinate (SR) and dehydroabietic acid. The analytical protocol developed (*all in one* wavelengths-retention time) for common abietanes present in Cuban colophony is simple, time-saving, eco-friendly, employing robust reversed-phase HPLC and offer the possibility to determine and quantify the main diterpenic acids (resinic acids) in the natural and modified mixture. The catalytic synthesis of dehydroabietic acid (DHAA) as a principal active pharmaceutical component from colophony for the potential treatment of neuropsychiatric dysfunctions and generation of exogenic cannabinoid analogues has been developed under ecological conditions using piritic ash as re-usable catalyst (disproportionation-aromatization) at meso-scale with minimal environmental impact. The neuropharmacological profile (including acute oral toxicity) of SR in rodent behavioural tests was determined; SR reduced spontaneous locomotor activity and aggressive behaviour, increased the number of sleeping animals and prolonged the thiopental-induced sleeping time indicating a sedative effect of test compound and it might not be related via the GABA or glycine systems. The SR is unable to protect animals against convulsion and death induced by pentylenetetrazole and picrotoxin. The SR (2000 mg/Kg p.o) didn't cause significant toxic symptoms in rats. This finding indicates that the SR can constitute a non conventional source of pharmacological molecular entities with central nervous system depressant activity. The synthetic DHAA has a sedative action on CNS and its acute toxicity ($DL_{50} > 5000$ mg/Kg) reveals that DHAA had not any toxic effects at dose concentrations used and could be employed as starting design structural point for developing molecular entities and series leads with potentially remarkable pharmacological properties.

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