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Safety Evaluation of the Dichloromethane Extract of *Hysterionica pinifolia*: A Potential Raw Material for the Development of Biopesticides

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Abstract

At the moment, there is a growing concern about the negative effects on human health and the environment due to the widespread and indiscriminate use of conventional insecticides. Since plants have been used throughout history to control microorganisms and insects, the safety is an aspect that must also be evaluated to guarantee that its use does not affect human health and the environment. The aim of the present study was to evaluate the safety of the dichloromethane extract of *Hysterionica pinifolia*, species with insecticidal activity, to be employed as a raw material for the development of biopesticides. The acute and subacute toxicity and the dermal and ocular irritation were evaluated. In these studies, the dichloromethane extract of *H. pinifolia* showed no ocular and a slight dermal irritation. Oral acute toxicity was greater than 2000 mg/Kg and less than 5000 mg/Kg (slightly hazardous) and no signs of toxicity were observed at repeated doses of 100 mg/kg body weight. These promising results showed that *Hysterionica pinifolia* could be considered as a potential raw material for the development of an insecticide from natural sources, safe for human health and the environment.

Keywords

Hysterionica pinifolia, Oral Acute Toxicity, Dermal Irritation, Ocular Irritation, Repeated Dose Toxicity

1. Introduction

There is growing concern about the negative effects on human health, environment and non-target organisms due to the widespread and indiscriminate use of

conventional insecticides [1]. For example, certain insecticides have negative effects on the nervous, renal, respiratory, and reproductive systems of men and women. Epidemiological studies have shown the association between exposure to pesticides and the risk and incidence of cancer [2] [3] [4] [5]. In addition to the detrimental impact of synthetic insecticides on human health, their excessive or inappropriate use is also linked to contamination of water, soil, and air, resistance of some pests to pesticides, and the reduction of biodiversity, among other factors [5] [6] [7]. The potential adverse effects associated with the use of chemical pesticides have also stimulated the research of new alternatives of pesticides from natural sources. In this sense, plants are a safe and efficient source of compounds with less toxicity for mammals, low persistence in the environment, and biodegradability [8] [9]. Currently, several studies have confirmed many of the beneficial biological activities attributed to them, such as insecticide, fungicide and repellent [10] [11] [12] [13]. However, the potential toxic or deleterious effects that their use could induce in short, medium or long term in human health and environment must be evaluated taking into account the potential routes of exposure for human beings [14].

Hysterionica pinifolia (Poir.) Baker (Asteraceae) is a perennial subshrub, native to the Argentinian flora. It grows in hilly areas of the province of Buenos Aires, between 0 and 800 meters above of sea level and in other South American countries such as Brazil and Uruguay [15]. In previous work the CH_2Cl_2 (dichloromethane) extract of this species showed insecticidal activity against *Tribolium castaneum*, a stored grain pest [16]. Regarding the chemical composition of *H. pinifolia*, acetylenic compounds were reported [16] [17]. *Cis* lachnophyllum methyl ester and the acetylenic alcohol (E)-undec-3-en-5,7-diyne-1-ol were identified as the major compounds in the active CH_2Cl_2 extract [16].

Polyacetylenes are widely distributed among Asteraceae, Araliaceae, and Umbelliferae families [18]. Some of the polyacetylenes isolated from food plants such as carrots, celery and parsley have various biological activities, like anti-inflammatory, antibacterial, antifungal and anticancer. According to their chemical structures, other polyacetylene compounds have shown toxic effects on humans and animals [19]. Some of these polyacetylenes are potent skin sensitizers and have shown cytotoxic and neurotoxic activities [20] [21] [22].

Considering the presence of compounds with possible toxic effects in the active CH_2Cl_2 extract from *H. pinifolia*, the development of a new insecticidal formulation using this extract as raw material requires a study of its safety.

2. Materials and Methods

2.1. Plant Material

Aerial parts of *Hysterionica pinifolia* were collected in Tandil, Buenos Aires province (west 59.1369, south 37.3286), Argentina, in March 2016 and was authenticated by Dr. Gustavo Giberti. A voucher specimen (BAF 858) was deposited in the Museum of Pharmacobotany of the Faculty of Pharmacy and Bio-

chemistry, University of Buenos Aires, Argentina. The material was dried at room temperature and finely powdered.

2.2. Extraction

The powered aerial parts (100 g) were extracted at room temperature by maceration using CH_2Cl_2 (1000 ml) for 1 h under continuous shaking. This procedure was repeated 5 times changing the solvent each time. These extracts were gathered. After evaporation of the solvent, 4.6 g of CH_2Cl_2 extract was obtained.

2.3. Animals

Healthy New Zealand rabbits and female and male Swiss mice and Wistar rats were used, following international guidelines and local regulations concerning to the care and use of laboratory animals for biomedical research (NIH Publication N˚ 85-23, Revised 1985). The study was approved by the Institutional Animal Care and Use Committee of the Faculty of Pharmacy and Biochemistry, University of Buenos Aires (Ethics approval: Exp-FFyB 0077054/17). The animals were acclimatized to laboratory conditions for 7 days. Rats and rabbits were housed in groups of five and individually, respectively. Animals were maintained in standard conditions (21˚C ± 2˚C; 12 h light/dark cycle). The animals were fed with a standard diet and tap water *ad libitum*.

2.4. Rabbit Skin Irritation Test

The test was carried out based on OECD guideline No. 404 [23] with slight modification. Approximately 24 h before the test, fur of the dorsal area of three rabbits was removed. 0.5 g of the CH_2Cl_2 extract was applied uniformly on area of 6 cm^2 of intact and abraded skin and covered with a gauze patch. Rabbits were examined for signs of erythema and oedema and the responses scored at 24, 48 and 72 h, and 15th day thereafter.

2.5. Rabbit Eye Irritation Test

The test was carried out by employing OECD guideline No. 405 [24]. Eye irritation was evaluated in 3 rabbits. Each one was instilled with 0.1 mL of the extract solution in the conjunctival sac of the right eye and the eyelid was immediately closed gently for a few seconds. The left eye was used as a control. The CH_2Cl_2 extract was evaluated at a concentration of 0.5 g/5mL dissolved in corn oil. The effect of the instilled sample on cornea (degree of opacity and area involved), iris and conjunctiva (redness, edema and secretions) were evaluated. Observations were made at 1, 24, 48, 72, 96 h and 7 days after the application of the sample. With the results obtained, the ocular irritation index was calculated.

2.6. Acute Toxicity in Mice

The evaluation of acute toxicity was carried out following the general guidelines of

OECD No. 420 [25] modified. Thirty mice, 15 males and 15 females, were randomly distributed into three groups of 10 animals each (5 males and 5 females). The CH_2Cl_2 extract was administered as an oral dose of 2000 or 5000 mg/kg body weight dissolved in corn oil. The control group for both sexes (5 males and 5 females) received corn oil. The administration was carried out by gastric tube. Mice were fasted for 4 h prior to dosing the extract. Mortality and any signs of toxicity were monitored twice a day (in particular, during the first 30 minutes and up to 4 h after administration) for 14 days. During this period, the body weight was recorded.

2.7. Repeated Dose Toxicity in Rats

The study was conducted following OECD guidelines 407 [26]. Thirty rats (15 male and 15 female) were randomly divided into three groups of 10 animals each (5 males and 5 females). The CH_2Cl_2 extract, dissolved in corn oil, was orally administered once a day at 100 mg/kg body weight to the treatment group for 35 days, while the control group received corn oil. The animals were observed for any signs of toxicity throughout the experimental period. Feed consumption, body weight and behavior were measured daily.

At the end of the experiment, the animals were weighed. Blood samples were collected for haematological study and for clinical biochemistry determinations. Haematological parameters were evaluated such as red blood cells, hemoglobin, hematocrit, total leukocytes and platelets. Glucose, total cholesterol, creatinine, uremia, glutamic oxalacetic transaminase (GOT), glutamic pyruvic transaminase (GTP), alkalinephosphatase (AP) were also evaluated.

A gross necropsy observation was carried out to find the presence of lesions on the external surface of the body, skeletal systems, body cavities (such as cranial, thoracic, abdominal and pelvic), as well as all orifices [27]. Liver, kidney, spleen, heart and ovary/testicles were excised free of fat and observed for gross pathological changes. The organ weights were recorded.

Histological studies of liver, kidneys, heart and spleen taken from all animals of each group were performed. They were fixed in 10% formalin in phosphate buffer, dehydrated in ethanol and finally embedded in paraffin wax. Histological sections of 5 μm thickness were obtained and subsequently stained with hematoxylin and eosin (HE) and were viewed under light microscope at 4×, 10× and 40× (Carl Zeiss Axioskop 2 Plus).

2.8. Statistical Analysis

The results were expressed as the mean ± standard error of the mean (SEM). An analysis of variance (ANOVA) was performed followed by the Dunnet test. $p < 0.05$ was considered a significant difference.

3. Results

3.1. Rabbit Skin Irritation Test

The skin's rabbit showed very slight erythema, but a reversion of this effect was

observed in two rabbits within 48 h. Oedema, alopecia, hyperkeratosis and hyperplasia were not observed on normal or injured skin in any animals.

3.2. Rabbit Eye Irritation Test

No ulceration, hemorrhage, opacity, swelling, tearing, oedema, redness and secretions were observed in the cornea, iris and conjunctiva during the observation period after instillation of the CH_2Cl_2 extract of *H. pinifolia*. The response to the light stimulus was not altered compared to the control eyes. The assigned score was zero, therefore the extract can be classified as non-irritating.

3.3. Oral Acute Toxicity in Mice

Although acute toxicity test revealed that oral administration of a single dose of 5000 mg/kg induced 80% mortality, dose of 2000 mg/Kg of the extract did not show any signs of toxicity or deaths during the 14-day observation period. No significant changes in body weight were observed (Table 1). The necropsy showed no macroscopic alterations in the liver, kidney, spleen and heart and no significant difference in the organ weight compared with control group (Table 1). These results showed that lethal dose 50 (LD_{50}) is greater than 2000 mg/kg and less than 5000 mg/kg of body weight.

Table 1. Acute toxicity of oral administration of the CH_2Cl_2 extract of *H. pinifolia* on body and organs weights on female and male mice.

	CH_2Cl_2 extract of *H. pinifolia* 2000 mg/kg	Control (corn oil)
Male		
Initial body weight (g)	26.28 ± 0.76	25.18 ± 0.65
Final body weight (g)	30.2 ± 1.20	29.82 ± 1.09
Liver (g)	1.328 ± 0.152	1.371 ± 0.079
Spleen (mg)	111.3 ± 6.1	92.42 ± 4.85
Kidney (mg)	405.9 ± 14.3	426.5 ± 19.3
Heart (mg)	180.4 ± 3.3	161.8 ± 11.3
Female		
Initial body weight (g)	21.14 ± 0.62	22.56 ± 0.36
Final body weight (g)	27.42 ± 0.37	27.16 ± 1.00
Liver (g)	1.193 ± 0.03	1.073 ± 0.089
Spleen (mg)	114.8 ± 6.2	119.0 ± 8.1
Kidney (mg)	311.1 ± 8.1	310.9 ± 10.3
Heart (mg)	173.1 ± 18.7	193.5 ± 17.3

Values expressed as mean ± SEM (n = 5 per group). No significant differences were observed with respect to the control group at $p < 0.05$.

3.4. Oral Repeated Dose Toxicity in Rats

The administration of the CH_2Cl_2 extract of *H. pinifolia* did not produce adverse effects in rats in the repeated dose safety evaluation test. During the evaluated period (35 days), body weight changes were comparable between treatment and control group. The consumption of food and water were similar between the rats that received the CH_2Cl_2 extract of *H. pinifolia* (100 mg/Kg/day) and the control group (Table 2). Furthermore, the extract did not produce behavioral changes or any other sign of toxicity at the evaluated dose (100 mg/Kg/day). In the macroscopic observations made in the autopsy, no visibly relevant differences were found in the main organs evaluated, no significant changes in organ weight (liver, heart, spleen, kidneys, ovaries or testicles) between the animals treated with the extract and the control group (Table 2). All the analyzed organs showed similar macroscopic and microscopic characteristics. Treatment with the extract did not induce significant changes in biochemical and hematological parameters, except in the group of males treated with the extract that presented a low value of hemoglobin and a low level of urea compared to the control group (Table 3).

Histological study

Histological examinations of the liver, kidney, spleen and heart of the group of

Table 2. Body and organs weights of the rats treated orally with the CH_2Cl_2 extract of *H. pinifolia* for 35 days.

	CH_2Cl_2 extract of *H. pinifolia* 100 mg/kg/day	Control (corn oil)
Male		
Initial body weight (g)	282.0 ± 7.6	300.0 ± 8.5
Final body weight (g)	379.0 ± 16.17	389.2 ± 6.094
Liver (g)	10.93 ± 0.6302	10.90 ± 0.2910
Spleen (g)	0.6977 ± 0.03620	0.6696 ± 0.02602
Kidney (g)	2.983 ± 0.1436	2.779 ± 0.07524
Heart (g)	1.379 ± 0.06155	1.466 ± 0.06766
Testicles (g)	3.400 ± 0.1612	3.469 ± 0.07481
Female		
Initial body weight (g)	177.4 ± 3.2	178.3 ± 3.1
Final body weight (g)	235.6 ± 3.3	230.0 ± 3.5
Liver (g)	6.917 ± 0.2011	5.876 ± 0.6523
Spleen (g)	0.5467 ± 0.04816	0.5067 ± 0.01746
Kidney (g)	1.938 ± 0.08555	1.798 ± 0.03302
Heart (g)	0.9789 ± 0.02448	0.9715 ± 0.02246
Ovaries (g)	0.1010 ± 0.198	0.1086 ± 0.01054

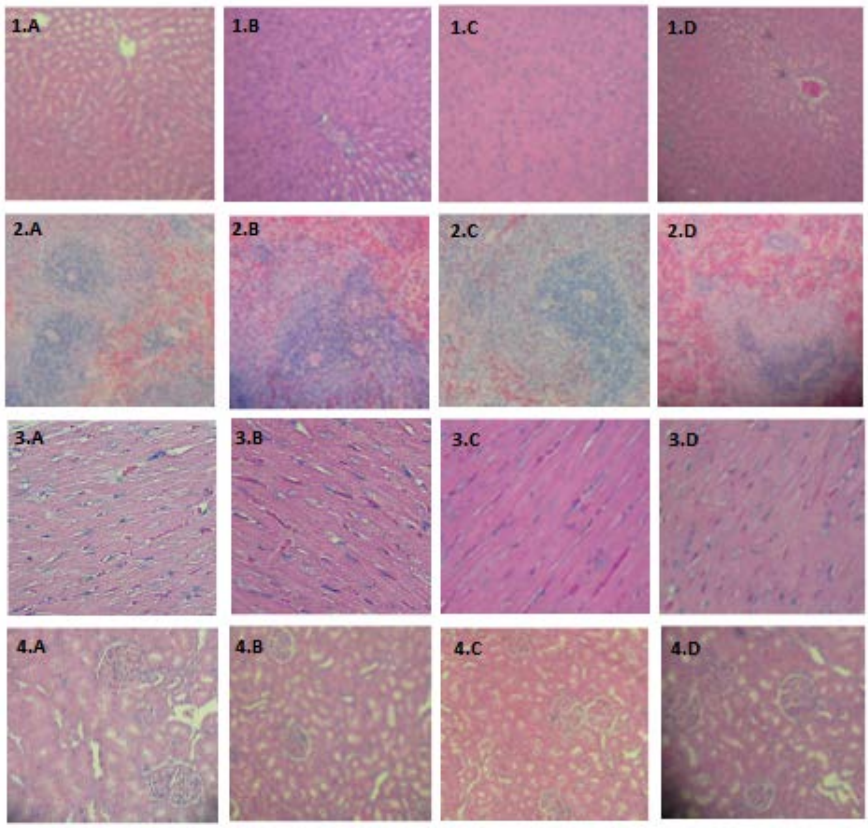
Values expressed as mean ± SEM (n = 5 per group). No significant differences were observed with respect to the control group at $p < 0.05$.

Table 3. Biochemical and haematological parameters of the rats treated orally with the CH_2Cl_2 extract of *H. pinifolia* for 35 days.

	CH_2Cl_2 extract of *H. pinifolia* 100 mg/kg	Control (corn oil)
Male		
Cholesterol (mg/dL)	46.80 ± 2.311	50.20 ± 1.655
Blood glucose (mg/dL)	122.4 ± 1.600	116.6 ± 1.990
Creatinine (mg/dL)	0.3960 ± 0.009274	0.4780 ± 0.04684
Uremia (mg/dL)	29.20* ± 1.114	37.60 ± 3.709
GOT (UI/L)	304.8 ± 125.2	268.8 ± 22.76
GTP (UI/L)	108.4 ± 58.52	62.60 ± 3.829
AP (UI/L)	114.8 ± 11.46	110.2 ± 11.74
Red blood cells (mm^3)	8,892,000 ± 192,760	9,280,000 ± 579,215
Leukocytes (mm^3)	8966 ± 835.0	8096 ± 469.4
Hemoglobin (%)	15.46*± 0.1364	16.44 ± 0.4020
Hematocrit (%)	48.60 ± 0.4000	51.40 ± 1.435
Platelets (mm^3)	778,400 ± 79,244	929,800 ± 92,341
Female		
Cholesterol (mg/dL)	61.20 ± 1.908	63.80 ± 3.541
Blood glucose (mg/dL)	112.0 ± 3.450	114.2 ± 3.917
Creatinine (mg/dL)	0.4480 ± 0.01625	0.4540 ± 0.01661
Uremia (mg/dL)	37.00 ± 1.483	34.20 ± 1.772
GOT (UI/L)	168.2 ± 13.76	163.4 ± 11.27
GTP (UI/L)	41.20 ± 1.463	43.00 ± 2.302
AP (UI/L)	55.00 ± 2.000	63.40 ± 8.964
Red blood cells (mm^3)	8,400,000 ± 143,324	8,110,000 ± 245,398
Leukocytes (mm^3)	8636 ± 1063	6934 ± 953.4
Hemoglobin (%)	15.48 ± 0.1594	14.62 ± 0.3105
Hematocrit (%)	48.26 ± 0.3742	46.00 ± 1.095
Platelets (mm^3)	741,800 ± 73,480	976,600 ± 143,192

Values expressed as mean ± SEM (n = 5 per group). *Significant difference compared to the control group at $p < 0.05$.

rats that received CH_2Cl_2 extract of *H. pinifolia* (100 mg/Kg/day) showed histo-structure similar to control group. Pathological changes were not observed in any group. No evidence of necrosis and inflammation were observed in any group (**Figure 1**).



A: male control; B: female control; C: male treated; D: female treated.

Figure 1. Histology of liver (1), spleen (2), heart (3) and kidney (4) of control and *H. pinifolia* (100 mg/Kg/day) treated animals.

4. Discussion

The principal aim of evaluating the safety of any medicinal plant is to identify the nature and significance of adverse effect and to establish the exposure level at which this effect is observed. In the search for safe insecticides for human health and the environment, *H pinifolia* was studied. In previous work, the CH_2Cl_2 extract of this species has demonstrated promising insecticidal activity and acetylenic compounds were identified [16]. The purpose of evaluating safety is not only to identify a potential adverse effect but also the significance of this effect. Considering the lack of toxicity reports of this species and the compounds identified in it, the safety profile of the CH_2Cl_2 extract must be studied in order to develop a safety insecticidal formulation. In this sense, dermal and ocular irritation, oral acute toxicity and repeated dose safety were evaluated.

Skin and eyes are high risk of exposure to insecticides during their manufacture but also in their use. The hazards may be meaningful not only in case of a single accidental exposure but also in many short and smaller exposures during a continued period of time, so information related to potential skin and eye irritation is a fundamental part to identify the potential risk of the products [28]. In this sense, it is important to determine if a substance or formulation for insecti-

cide use could induce skin and eye irritation to be properly classified and labeled as recommended by Good Agricultural Practices and, if it is necessary, guarantee the appropriate use of personal protective equipment (protective glasses, face shields) [29] [30].

In the study of primary dermal irritation, the CH_2Cl_2 extract of *H. pinifolia* presented a slight dermal irritation with barely perceptible erythema formation and no edema formation. Due to the CH_2Cl_2 extract of *H. pinifolia* did not produce corrosion or severe skin irritation, eye irritation was evaluated, showing that the extract did not produce signs of irritation during observation period.

Besides considering that exposure could occur when toxic substances get into de body through mouth, the oral route represents a potential route of accidental human exposure. Therefore, the determination of acute and repeated dose toxicity allows the hazard classification of test substances [31]. In this sense, acute oral toxicity was determined for the CH_2Cl_2 extract of *H. pinifolia* at 5000 mg/kg and 2000 mg/kg. The initial dose (5000 mg/kg) was selected adopting the guidelines of the World Health Organization (WHO) (2019) [32] for the toxicological classification of pesticides according to risk and LD_{50}. In this investigation, the acute oral toxicity of the CH_2Cl_2 extract of *H. pinifolia* was greater than 2000 mg/Kg and less than 5000 mg/Kg. Therefore, according to the WHO toxicological classification (2019), it is considered slightly hazardous (Category III).

The toxic effects observed in the acute oral toxicity at the concentration of 5000 mg/Kg and the slight dermal irritation could be attributed to the presence of acetylenic compounds in the CH_2Cl_2 extract. In this sense, *cis* lachnophyllum methyl ester and the acetylenic alcohol (E)-undec-3-en-5,7-diyne-1-ol, were identified as major compounds in the CH_2Cl_2 extract of *H. pinifolia* [16]. Although there are no reports of toxicity of these identified acetylenes, other compounds with analogous structure showed acute toxic effects, contact dermatitis and irritant skin reactions for humans and animals. For example, cicutoxin and oeantotoxin isolated from *Cicuta virosa* L. (Apiaceae) and *Oenanthe crocata* L. (Apiaceae) respectively, showed acute toxic effects in humans and domestic animals [22]; the acetylenic alcohol falcarinol is responsible for most allergic skin reactions caused by plants of the Apiaceae and Araliaceae families, while structurally related polyacetylenes such as falcarindiol and falcarinone had no irritating effect on the skin [18] [22].

In view of repeated exposure, toxicological evaluations are vital to characterize the toxicological profile of xenobiotics [33]. In this work, the analyzed parameters showed promising results.

Changes in body weight as well as organ weight have been used as an indicator of potential adverse effects of chemical compounds, such as tubular hypertrophy, progressive nephropathy or hepatocellular hypertrophy related to changes in kidney or liver [34] [35]. The administration of repeated doses of the CH_2Cl_2 extract of *H. pinifolia* did not produce changes in the body weight of the ani-

mals, neither in the weight of the organs evaluated.

Histopathological analysis of organs and tissues represents a way to evaluate possible pathological changes related to the administration of repeated doses of a certain substance. The observation of cellular structures allows to determine pathological alterations with greater precision [33]. In the studied organs, a normal cellular morphology was observed, without evidence of necrosis and inflammation between the groups that received daily the CH_2Cl_2 extract of *H. pinifolia* compared to the corresponding control groups.

On the other hand, considering that level of glucose and lipids can detect alterations in the metabolism [36], these parameters were measured. The values of blood glucose and cholesterol did not present significant differences between the treated and control groups.

Creatinine, uremia, GOT, GTP and AP are used as biomarkers of renal or hepatic injuries [33]. In this study, no statistical differences in biochemical parameters were observed between the treated and control groups. Only, uremia in males treated with the CH_2Cl_2 extract showed a statistically significant decrease compared to the control group, nevertheless the observed level of uremia (29.20 ± 1.114 mg/dL) lacks biological significance since it is not below 20 mg/dL, a value that indicates hypo-azotemia.

Also, changes in hematological parameters have been used to predict toxic effects [37] but the administration of repeated doses of the CH_2Cl_2 extract of *H. pinifolia* did not affect the evaluated hematological parameters.

The results obtained in the repeated dose safety test in rats, showed that the administration of the CH_2Cl_2 extract of *H. pinifolia* did not produce significant adverse effects. The employed dose (100 mg/kg/day) is six times greater than the EC_{50} of the extract obtained in the insecticide activity bioassay on *T. castaneum* [16]. Therefore, it could be expected that the use of the CH_2Cl_2 extract of *H. pinifolia* at the EC_{50} dose would be safe for human health.

5. Conclusions

In this study, the CH_2Cl_2 extract of *H. pinifolia* presented a slight dermal irritation, no ocular irritation, dose of acute oral toxicity is between 2000 mg/Kg 5000 mg/Kg (slightly hazardous) and no signs of toxicity at repeated doses of 100 mg/kg body weight.

The promising results of insecticidal activity obtained in previous works and the safety results obtained in this study with the CH_2Cl_2 extract of *H. pinifolia*, allow us to consider this extract as a potential raw material for the development of an insecticide of natural origin, safe for human health and the environment.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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