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Purification of a Natural Insecticidal Substance from *Cestrum parqui* (Solanaceae)

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Abstract: *Cestrum parqui* is a shrub originating from Chile used in Tunisia as an ornamental plant. The toxicity of this plant was observed for the first time on the Desert Locust *Schistocerca gregaria*. It was shown that the toxicity of the plant is concentrated in the Crude Saponic Extract (CSE). In the present research we try to study chemically this fraction to isolate the active substances. The CSE was separated by column chromatography and the obtained fractions were biologically tested on *Schistocerca* larvae. We observed that only the fraction F9 was active, the separation of this fraction on preparative plates permits to isolate the active compound called S1. The tentative of structure elucidation of the natural substance S_1 using ^1H and ^{13}C NMR shows that S_1 is an heterosidic saponin. Structural modification of S_1 structure provoke its activity lose.

Key words: *Cestrum parqui*, *Schistocerca gregaria*, insecticide, saponin

INTRODUCTION

Synthetic insecticides (i.e., organophosphorus, organochlorine, carbamates) causes many toxicological and ecotoxicological problems. Many alternative methods of plant pest control were developed. The plants have an interesting source of pesticidal active molecules. Certain plants secrete pesticide or pest repellant substances. The chemical and biological study of these substances can be the source of new natural pesticides development.

Cestrum parqui is a shrub originating from Chile used in Tunisia like ornamental plant. The toxicity of this plant was shown for the first time on the desert locust *Schistocerca gregaria* (Ammar *et al*., 1995). This plant is also toxic for some other Lepidoptera (*Spodoptera littoralis*, *Helicoverpa armigera*, *Pieris brassicea*) (Chaieb *et al*., 2001, 2004). Some antecedent works shown the molluscisidal activity of this plant on *Theba pisana* a crop devastating snail (Chaieb *et al*., 2005).

It was shown that the toxicity of the plant is concentrated in the Crude Saponic Extract (CSE) (Barbouche *et al*., 2001). It is interesting to study chemically this fraction to try to isolate the active substances and to explore their pesticidal potential.

MATERIELS AND METHODS

Animals rearing: The insect's eggs are brought from a breeding of desert locust *Schistocerca gregaria* at the gregarious form maintained in the laboratory of physiology and physiopathology of the insects of INAT (National Tunisian Agronomic Institute), this insect is maintained since the last great invasion of the locust in 1988-1989.

The eggs are laid in plastic cups filled with sand constantly moistened with water to avoid their drying. These eggs are maintained in fixed room temperature (30°C), in small wooden cages of 20×30×40 cm in a glazed greenhouse cell provided with a cooling system. The breeding is maintained in strong population (300 to 500 individuals per cage). The food consists of grass and cabbages leaves.

Adults are kept in wood cages 50×50×50 cm which are provided with 7 cm of diameter plastic cups serving for laying eggs. The breeding of the adults is done under the same conditions described previously (glazed greenhouse). The adults or the larvae undergoing of the experiments are maintained in laboratory conditions.

Saponins extraction: The saponin extraction method is described by Barbouche *et al*. (2001). The leaves of

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*C. parq*ui was obtained from the garden of National Tunisian Agronomic Institute, dried in a steamroom at 40°C during 4 days, the dried leaves are finely grounded. 100 g of the powder washed with petrol ether, then extracted three times with 300 mL methanol. After filtration the methanol is evaporated with rotary evaporator At 40°C. We obtained a dry residual weighing 6 g, the dissolution of 1 g of this residual in 100 mL methanol then the addition of 100 mL of ethylic ether permits to get 0.06 g of a brown precipitate symbolized CSE (Crude Saponic Extract).

Chromatographic techniques

Thin layer chromatography: Extracts and substances are deposited with capillarity tube on TLC. If the components of the sample are coloured, their separation is easily observable. In the contrary case, we must make the spots visible by a process of revelation.

The observation of the spots is done with UV at 254 and 312 nm or revealed either by ammonium molybdate dissolved in a water-sulfuric acid mixture (20 g of ammonium molybdate + 40 mL of H_2SO_4 + 400 mL of distilled water) or with a mixture H_2SO_4/MeOH (10%) followed by heating.

Chromatography on preparative plates: The same principle as the TLC except that in this case the stationary phase is thicker and on which we can deposit and separate more important quantities of products. The detected bands are scraped using a spatula then separated from silica using a suitable solvent. The mobile phase used in this case is described by Baqai *et al.* (2001) it is a mixture of acetic acid-water and butanol.

Column chromatography: Like thin layer chromatography, this technique based on the separation of the organic substances on a solid phase as silica gel 60 SDS F 254 0.063-0.2. The elution is done by solvents of increasing polarity. Fractions of 200 mL are collected and the solvent is evaporated using a rotary evaporator. The complexity of the fractions obtained is tested on TLC plate. The fractions having the same allure are mixed. Coarse columns (1 m length, 5 cm of diameter) are used for the rough extracts and of the columns of small size (40 cm length, çm of diameter) are used for finer separations.

Grafting of extract on silica gel: When the studied extracts did not have a suitable solubility in the mobile phase used for the column, the tests have been carry out a grafting of a small quantity of silica gel by the extract in question. In the case of CSE, 5 g of this extract was dissolved with methanol then mixed with 40 g of silica gel, the solvent was completely evaporated with a rotary evaporator. The grafted silica gel was then deposited by small portions on the higher part of the column containing silica and overcome by the mobile phase.

Structural modification of saponins

Saponins hydrolyse: It is a reaction permitting to cut the link sugar-O-genin, this permits to test the activity of the aglycone moieties. This reaction is done in acid medium. 0. 5 g of CSE solubilized in methanol is placed during 24 h under backward flow in the presence of 4 mL HCl (10%). The extraction of the hydrolysed genins is done by chloroform. The drying of the organic phase with anhydrous Na_2SO_4 then the evaporation of solvent using a rotary evaporator lead to obtain the genins moieties in solid form.

Saponins acetylation: Acetylation is a reaction permitting to esterify any primary and secondary alcohol group. Tertiary alcohols are difficult to esterify because of steric obstruction. This reaction is done in pyridine with acetic anhydride. Indeed 0.5 g of CSE dissolved in 3 mL of pyridine and treated by 3 mL acetic anhydride. The mixture is left under agitation during 48 h at the ambient temperature. A dilution with the distilled water of the reactional mixture and an extraction with the chloroform followed by drying on Na_2SO_4 anhydrous and evaporation make it possible to obtain the acetylated saponic fraction.

Biological test: Insecticidal activity is carried out using a grafting technique. This operation consists in introducing a substance in solid form under the cuticle of the insect, this technique is practiced when we cannot dilute the tested substance in water and when the organic solvents are toxic for the insect (Table 1).

The L_5 larva of *S. gregaria* are incised laterally on the level of the 3rd abdominal segment. The incision is done along the segment (3 mm approximately). A CSE crystal of approximately 5 mg is inserted in the incision. A paraffin drop is deposited on the incision, to obliterate the wound and to prevent the loss of the hemolymph. The

Table 1: Results of biological test of different obtained fractions on *Schistocerca* larvae

Group of fractions	Animal used	Mortality	Activity
F_1	-	-	Not tested
F_2	5	0	Inactive
F_3	5	0	Inactive
F_4	1	0	Inactive
F_5	2	0	Inactive
F_6	5	0	Inactive
F_7	4	0	Inactive
F_8	2	0	Inactive
F_9	5	5	Active

number of used individuals depends to the available quantity of tested substance, generally substance or extract is considered to be active when we have 4 mortalities on 5 tested insects.

RESULTS

In order to have an idea on the complexity of the crude saponic fraction and choose the adequate system of elution to use during separation on column, we analyzed this dissolved fraction in methanol on TLC plate using $CHCl_3$/MeOH 1:1 mixture as mobile phase.

TLC Plate (Fig. 1) revealed by molybdate makes it possible to visualize a spot with a high FR (frontal report ratio) (Spot 1) which corresponds to one or more relatively non-polar substances and a trail (Spot 2) which could correspond probably only to saponins constituting this extract. The adsorbent character of these natural heterosides is in agreement with the obtained chromatographic profile.

To eliminate the hydrophobic substances of he spot 1 it has been proceed to make several washings of the CSE extract with a mixture of hydrophobic solvents (dichomethane/petroleum ether 1:1). The residue of this washed phase and washed CSE were tested biologically on *Schistocerca gregaria*.

With an aim to isolate the active substances, the washed crude saponic extract was simplified on a silica gel column in 300 fractions of 200 mL each one using a mixture of methanol and ethyl acetate as mobile phase. The analysis of all these last fractions on TLC plate enabled us to gather them in nine fraction groups (F_1-F_9) (Fig. 2).

All fractions are evaporated and tested biologically on 1 to 5 *Schistocerca gregaria* L_5 larvae in correspondence with the disponibility of the fraction residue. The fraction is considered to be active when all tested individuals were dead. The F_1 fraction is not tested because of insufficient matter.

We observe that all fractions are inactive with the exception of F_9. This last was separated on preparative silica gel plates, we remark a major band that we can

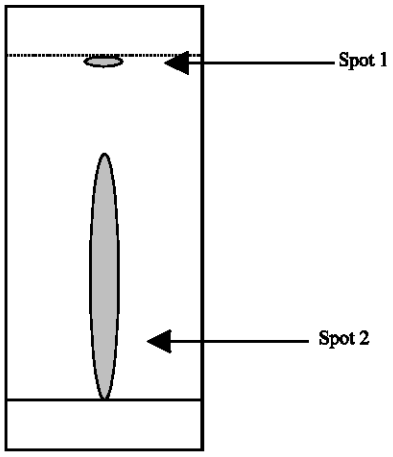

Fig. 1: TLC chromatogram of CSE eluated by $CHCl_3$/ Méthanol 1:1 mixture

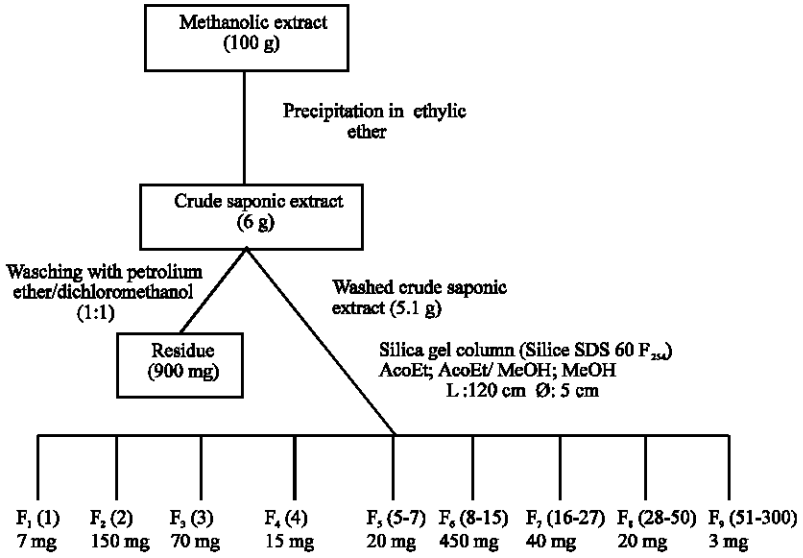

Fig. 2: Separation schema of the CSE

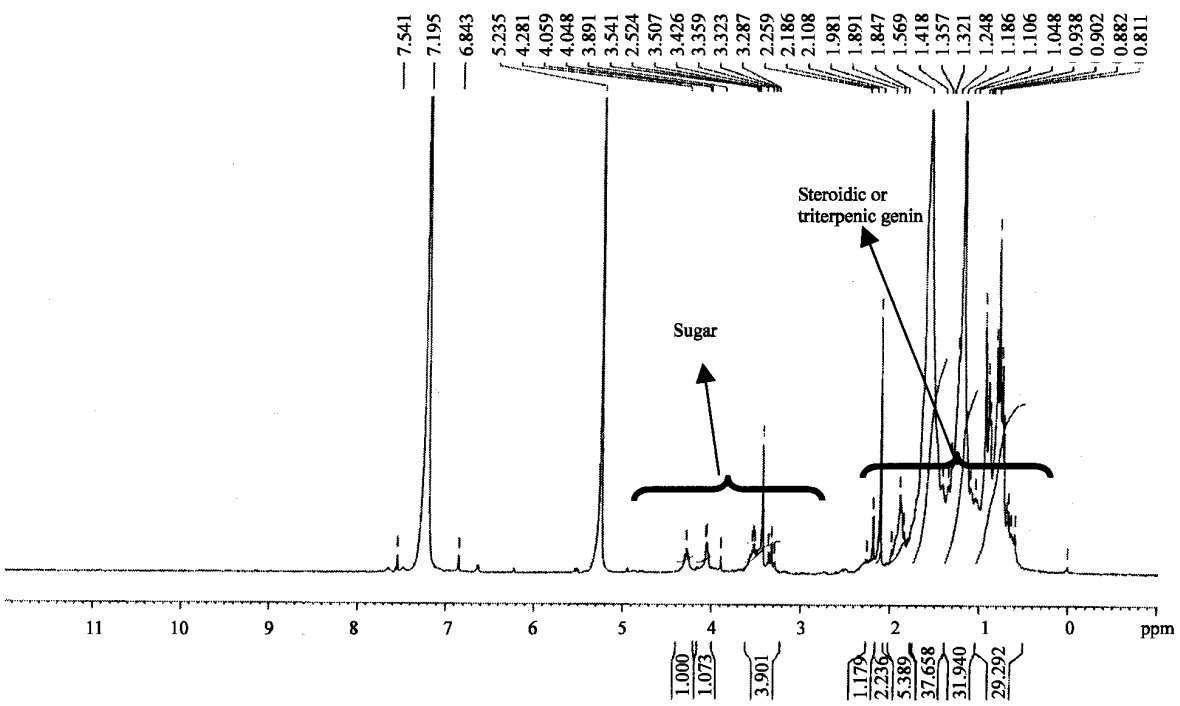

Fig. 3: ^{1}H RMN Spectra of S_1 (CD_3OD, 300 MHZ)

purify by scarping silica gel on plate. After filtration and evaporation we obtain a white solid substance called S_1; this substance is tested biologically and it shows high toxicity to *Schistocerca*.

A structural study of the isolated product S_1 was carried out by means of some spectroscopic techniques such ^{1}H and ^{13}C NMR.

S_1 ^{1}H NMR spectrum analysis: Analysis of the ^{1}H NMR spectrum of S_1 recorded with 300 MHZ in deuteried methanol (CD_3OD) (Fig. 3) allows to observe:

- A whole of signals between 0.5 and 2.5 ppm usually attributed to steroids and triterpenes protons.
- Some other signals in the zone 3-4.5 ppm generally correspond to saccharides protons.

These observations above permit to think that S_1 structure could be an heterosidic one.

S_1 ^{13}C NMR spectrum analysis: The study of the ^{13}C NMR spectrum of S_1 recorded with 75 MHZ in deuteried methanol (Fig. 4) consolidates an heterosidic structure with a steroid or a triterpene aglycone, this is due to the observation of:

- Several signals in the area with strong field between 10 and 50 ppm relating to the majority of carbons belonging to the steroids or triterpenes.
- A significant number of signals in the zone going from 60 to 105 ppm corresponding to saccharide carbons.

The complexity of the spectra recorded for S_1 did not enable us to determine the complete structure but to propose just the nature of the molecule. Other two-dimensional NMR are necessary to elucidate S_1 structure.

Effect of structural modification on the insecticidal activity: Biological tests are made using grafting tested substances under the cuticle of *Schistocerca*. Three batches of 10 adult males are used. The first batch receives a grafting of approximatively 5 mg of CSE crystal, the second a crystal of acetylated saponins and the third receives hydrolyzed saponins (usually 5 mg). A daily counting of mortality was carried out. Results are summarized in the Fig. 5.

We noticed according to this experiment that the insects treated with a crystal of CSE are all dead. It seems that the other batches do not suffer from neither acetylated nor hydrolyzed CSE (Fig. 5). This demonstrates the loss of the activity of saponins by these two modes of structural modification.

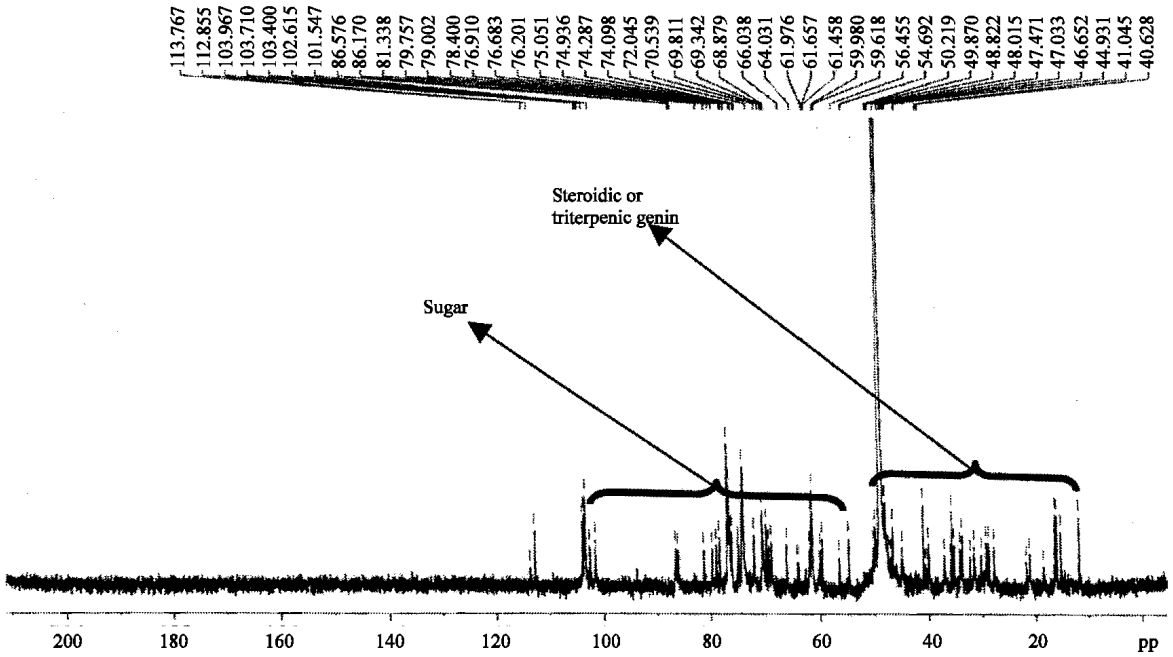

Fig. 4: ^{13}C RMN Spectra of S_1 (CD_3OD, 75 MHz)

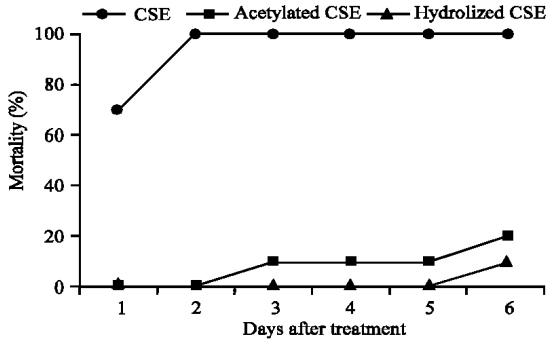

Fig. 5: Daily mortality evolution in *Schistocera* with different treatment

DISCUSSION

In this research the chromatographic study of *Cestrum parqui* CSE permits to isolate an active product symbolized S_1. The spectral exploration of this molecule reveals that it is an heterosid saponin. Many plant saponins are described to have insecticidal activity. Indeed, The pulverization of the plant leaves by 0.1 to 0.2% of saponins of alfalfa reduces phytophagous acarian number (*Tetranychus urticae*) and aphids (*Pharodon humulus*) respectively of 85 and 90%. Saponins of this plant can also cause mortalities on eggs of *T. urticae* (Oleszek *et al*., 1999; Puszakar *et al*., 1994). Saponins of alfalfa mixed with the food of *Ostrinia nubilalis* cause larval mortalities going up to 100% for the young larval stages. These mortalities also touch the nymphal stage, only 60% of the treated chrysalis emerge (Nozzolillo *et al*., 1997). Treated by 100 ppm of leave saponins, *Spodoptera littoralis* shows a cumulative mortality of 90% at the larval and nymphal stages (Adel *et al*., 2000). Moreover, it have been shown for the same insect various forms of chronic toxicity like a reduction in the fertility of the females and in the rate of eggs hatching (Adel *et al*., 2000). The saponins extracted from the leaves and the roots of the same plant are toxic for the larvae of *Leptinotarsa decemlineata* (Szczepanik *et al*., 2001).

The addition of Aginoside 1 (steroidic saponin) in the artificial diet of *A. assectella* larvae at a rate of 0.9 mg g^{-1} involves 56% of mortality in this insect (Harmatha *et al*., 1987).

The commercial saponins extracted from *Quillaja saponaria* have a larvicidal activity against the larvae of mosquito of two species *Aedes aegypti* and *Culex pipiens*. Hundred percent of mortality are obtained by using amounts of 1000 mg L^{-1} during 5 h (Pelah *et al*., 2002).

The biological tests relating with the modification of the structure of *Cestrum* saponins structure (acetylation and hydrolysis) demonstrates that in all the two cases we ended to a total loss of the molecules activity, which confirms results obtained by various researchers:

Barbouche (2001) reported that sapogenins of the *Cestrum* are less active than saponins, which pleads in favour of the loss of the activity of hydrolysed saponins. Indeed, we showed that the aglycone obtained was inactive by grafting of these crystals on *Schistocerca gregaria*. The same results where obtained by acetylated saponins. It seems that the various structural modifications would lead to a loss of the hydrophily of the molecule which would need the sugar chain for its solubility in the hemolymph of the insects or the culture media, this phenomenon deserves to be better studied in order to understand the mode of saponins action.

Moreover, various authors report the loss of the saponins biological activity by their structural modifications. Indeed, Keukens *et al.* (1995) showed that a reduction of the chain of α-tomatine or α-choacine involved a total loss of their activity (membrane rupture). In the same way, a study of the interaction digitonine/ cholesterol shows that the analogues of Digitonine could be complexed with cholesterol. Various degrees of digitonine glycosylation are undertaken: Two, four or five sugars are associated to the genin, complexation increases when we increase the number of associated sugars (Takagi, 1982).

Hu *et al.* (1996) then Armah *et al.* (1999) confirm these results by using similar saponins having triterpenic genin and by showing successively that the sugar nature influences slightly the general molecule activity, on the other hand the hydrolysis of one or more sugars involve the total or partial loss of this activity.

The partial hydrolysis of the sugar chain also seems to be a strategy of detoxification of saponins by phytopathogenic fungi. These fungi indeed secrete hydrolase enzymes which provoke loss of saponins activity by elimination of one or more sugars of the C_3 chain (Morrissey and Osbourn, 1999). It seems also that the genine itself plays a role in the total activity of saponins, indeed a structural modification of the genine involves the change of the membrane rupture activity of glycoalcaloidic saponin extracted from *Solanum* genus (Roddick *et al*., 2001).

The *Cestrum parqui* represents a high potential for isolating natural insecticide molecules, its saponins represent an excellent model for studying the pesticidal activity of this kind of substances. More studies are necessary to identify the active product and to explore its physiological mode of action.

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