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Antifeedant and repellent effects of neotropical *Solanum* extracts on drywood termites (*Cryptotermes brevis*, Isoptera: Kalotermitidae)

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Abstract

Antifeedant and repellent effects of two different extracts from native *Solanum* species, *S. bistellatum* and *S. sisymbrifolium* on *Cryptotermes brevis* were evaluated. The extracts obtained, particularly the dichloromethane extracts and the enriched fraction of sugar esters from both species, showed antifeedant and repellent activity against the termite. The antifeedant effect of dichloromethane extract from *S. sisymbrifolium* at the concentration of 25mg/mL reached 100%, while the repellent action of the dichloromethane extracts and the dichloromethane-acetone extract for sugar esters (enriched fraction of sugar esters) at 1mg/mL was greater than 90%. In the case of *S. bistellatum*, the antifeedant effect of the dichloromethane extract and dichloromethane-acetone extract for sugar esters at the concentration of 25.00mg/mL was 43% and 57%, respectively. The repellent action of the dichloromethane extracts and of the enriched fraction of sugar esters at a concentration level of 2.5mg/mL was greater than 92%.

Introduction

Solanaceae is a cosmopolitan plant family, being particularly varied and abundant in tropical and subtropical regions of Central and South America (Gutiérrez et al., 1999). They are known for producing a wide range of toxic molecules (Pomilio et al., 2008). Despite these secondary metabolites, some also have properties with low insecticidal activity, e.g., sugar esters (Tingey & Laubengayer, 1986; Hori et al., 2011). Particularly, species belonging to the genus *Solanum* produce a vast array of chemically diverse bioactive secondary metabolites. *Solanum* is heterogeneous and widely distributed in the New World (Vázquez, 1997). Most species of the genus have trichomes on stems, leaves, and inflorescences (Mentz et al., 2000) which produce chemical substances with anti-insect activity (Silva et al., 2003; Silva et al., 2005; Lovatto & Thomé, 2004; Leite, 2004; Srivastava & Gupta, 2007; Szafranek et al., 2008). Glandular trichomes from *S. berthaultii* have been used as morphological markers to denote resistance to aphids in new potato cultivars. These trichomes produce sugar esters that are well known for their anti-insect properties (Yencho et al., 1993).

The bioactivity of a new family of sugar esters isolated from *S. sisymbrifolium* type IV trichomes leaves was reported by Cesio et al. (2006). As *S. sisymbrifolium* is a widely distributed weed that produces relatively high amounts of such compounds, it was planned to evaluate the antitermitic activity of these compounds. The relationship between chemical family and biological activity deserves more in-depth investigation. Therefore, we searched Latin American *Solanum* species for leaf morphology and chemical composition of the obtained extracts that might yield secondary compounds with similar structural features like those described in *S. sisymbrifolium* (Cesio et al., 2006).

Termites are among the major pests of wood, and particularly the West Indian drywood termite, *Cryptotermes brevis* Walker (Isoptera: Kalotermitidae), which is endemic to Latin America (Constantino, 2002), and has been disseminated

unintentionally into many regions of the world in boats and wooden packaging material (Scheffrahn et al., 2009), damaging furniture, buildings, and even the wooden statues from Ouro Preto among other masterpieces in Brazil. The most widely used method to control termites is based on the application of chemical insecticides which might possess negative effects on humans and the environment (Cabrera et al., 2001; Stephenson & Solomon, 2007). Alternative control strategies, like biological control or the use of less toxic plant extracts, (Smith, 1989; Harborne, 2001) should be considered. Nevertheless, little bio-prospecting for active plant extracts against termites is reported in the literature despite the great potential that the unexplored flora of Latin America might possess.

Using a chemotaxonomic approach to find bioactive compounds from plants against termites, it was hypothesed that the Solanaceae family could be a source of new compounds due to its high chemodiversity. The objective of the present study was to assess the antifeedant and repellent effects of leaf extracts from *S. bistellatum* and *S. sisymbrifolium* on *C. brevis*, from the acyl sugars secreted by type IV trichomes.

Material and Methods

Plant Material

Solanum sisymbrifolium samples were collected in Montevideo, Uruguay, and *S. bistellatum* in Rio Grande do Sul, Brazil. The species were labeled as vouchers 3520 and 4327, respectively, and kept at the Jose Arechavaleta Herbarium in the Faculty of Chemistry, UdelaR, Uruguay.

Extracts

Three different extracts were obtained from each plant species, as follows: 1) Dichloromethane extract: 300 g of fresh leaves were immersed portion wise in 1000 mL of dichloromethane for 30s. The dichloromethane solutions were evaporated under reduced pressure to dryness. 2) Dichloromethane-acetone extract for sugar esters: 1g of the dichloromethane extract was dissolved in acetone (100 mL) and the solution was cooled to -20°C and kept overnight at this temperature. The resulting precipitate was discarded and the acetone extract was evaporated under reduced pressure to dryness to yield the extract containing the enriched fraction of sugar esters. 3) Aqueous-ethanolic extract: The remaining plant material from the first procedure was immersed in an ethanol/water (70:30) solution for 24h. The ethanol solution was filtered and evaporated under reduced pressure to yield the final extract.

Crude Extract Composition

Ultrapure reagents (Sigma Aldrich) were used in the analysis. Thin-layer chromatography (TLC) was conducted using Polygram Sil/UV 254 on 0.25-mm-layered plates (Macherey-Nagel).

NMR spectra were measured using a Bruker Advance 400MHz spectrometer. Standard pulse sequences were used for the different NMR experiments. Samples were dissolved in $CDCl_3$, (Sigma-Aldrich). Gas chromatography-mass spectrometry (GCMS) was performed with an HP 6890/6973 using an HP-5 25-m-long, 0.5 mm (I.D.) capillary column. The temperature program used was T_i=60ºC for 10 min, 10 ºC/min to T_f=280 and hold for 20min. The conditions of the MS quadruple were the standard. The compounds were identified using the NIST library with a SI > 90%. All solutions were evaporated under reduced pressure at ≤ 60°C.

Chemical Analysis

The phytochemical study was based on bioguided fractionation (Hostettmann, 1999) aiming to characterize the most bioactive fractions but also taking into account our previous findings on the bioactivity of sugar esters and the extracts that contain them (Cesio et al., 2006; Dutra et al., 2008). Thin-layer chromatography was performed with the different extracts obtained from *S. bistellatum* and *S. sisymbrifolium*, using CH_2Cl_2/MeOH (9:1) as the mobile phase. Compounds were detected by spraying TLC plates with a universal dye reagent of 5% $CuSO_4$ in 10% aq. H_3PO_4 and subsequent heating in oven at 20-140ºC (4ºC/min). Specific dyeing reagent used for sugars was diphenylamine, aniline, and acetone phosphoric acid (Anonymous, 1974).

The major compounds of *S. bistellatum* were isolated from the enriched fraction of sugar esters by column chromatography. Fifty mg of extract was seeded at the top of an open column packed with 10 g of silica gel (Macherey-Nagel MN, 60). Elution was performed using a solvent gradient of increasing polarity: 30mL of dichloromethane; 50mL of dichloromethane/methanol (9.5:0.5); 50mL of dichloromethane/methanol (9:1); dichloromethane/methanol (8.5:1.5). Fractions of 2mL were collected and their composition was confirmed with TLC using the same conditions stated above. The fractions containing the same compound were combined, the solvent was evaporated under reduced pressure, and structural elucidation using NMR analysis was performed. The fatty acid composition of the acyl sugars was investigated following the procedure described by Dutra et al. (2008). One to 10mg of the sugar ester was treated at room temp. with 0.5mL of 0.5N methanolic KOH for 15min. One mL of 1N methanolic HCl was added and the solution left at room temp. overnight. The precipitated KCl was then filtered off and the methanol evaporated to dryness. The residue was redissolved in AcOEt and analyzed by GCMS.

Insects

Cryptotermes brevis pseudergates were collected from wooden houses located in the state of Rio Grande do Sul, Brazil. The wood was collected and stored in plastic containers covered with cloth. The wood pieces were stored under appropriate conditions of temperature and humidity for termites (24 ± 1ºC and 70± 5% RH, respectively).

Bioactivity of Solanum spp. extracts on C. brevis

Bioactivity was assessed following the methodology described by Sharma and Raina (1998), with a focus on antifeedant and repellent activities. To obtain the required concentrations, the dichloromethane extract and the enriched fraction of sugar esters were diluted in acetone, while the aqueous-ethanol extract was diluted in water. In all assays, 1mL of the extract solutions was applied to filter paper discs (9.0cm diam.). Control groups consisted of filter paper discs treated with solvent only. To evaporate the solvent, discs were maintained under a laminar-flow hood for 48h.

The discs were then placed in Petri dishes to assess the behavioral response of 30 *C. brevis* pseudergates which were placed in the bioassay units. Bioassays were run for 30 days in five replicates under appropriate conditions of temperature and humidity for termites (24 ±1ºC; 70 ±5% RH; 24h scotophase).

Antifeedant Activity

Substrate consumption was determined by mass loss of filter paper exposed to the termites where: [(initial mass = post-impregnation with the product) – (final mass = post-exposure to the termites)]/Initial mass x 100. The difference in mass considered filter paper discs dried (60ºC) for 4h and weighed with an analytical balance. Feeding inhibition (FI) of each extract concentration (2.5, 12.5, and 25 mg/mL) was determined by the equation (FI%) = (control disc consumption – treated disc consumption/control disc consumption + treated disc consumption) x 100 (Simmonds et al., 1990).

The concentration per unit (mg/cm^2) area was calculated using the following formula:

C(mg/cm^2)=c(mg/mL)/Πr^2. In our case, values for the antifeedant assay were C= 2.5, 12.5, and 25 mg/mL/3.14159 x (4.5)^2 and for the repellent assay C= 2.5, 12.5, and 25 mg/mL/3.14159 x (4.5)^2.

Repellency

Repellency was assessed by gravimetric measurement of termite feeding on the treated or untreated paper. Filter paper discs were cut into two halves of which one-half was treated with an extract and the other half with solvent only. Thirty termites were placed in the middle of the plate and their location counted every four days for 30 days. The following concentrations of the extract were used: 0.25, 1, and 2.5 mg/mL. Repellency percentage (RP) was calculated daily using the following formula of measurement and finally the data pool was statistically calculated with: RP = (Ca – Ta) / (Ca + Ta) x 100, where Ca = number of termites present on control area and Ta = number of termites present on treated area. Negative values for RP were considered as null (McDonald et al. 1970). Statistical analysis was performed using parametric tests and SPSS software version 18.0 for Windows. Data were analyzed using two-way ANOVA followed by Tukey's test.

Results and Discussion

The antifeedant effect of extracts on *C. brevis* showed varying results. The aqueous-ethanol extract had little or no effect on filter paper consumption (Table 1), but both organic extracts showed significant antifeedant effects. The acetone fraction from the dichloromethane extract from *S. bistellatum* had a higher antifeedant effect than the crude dichloromethane extract, whereas the opposite was observed for the extracts obtained from *S. sisymbrifolium.*

The dichloromethane extract and the enriched fraction of sugar esters of *S. bistellatum* showed mild antifeedant activity (30-59%) on *C. brevis*. On the other hand, the aqueous-ethanol extract showed phagostimulant activity at concentrations of 12.5 and 25 mg/mL. The dichloromethane extract of *S. sisymbrifolium* inhibited feeding completely at 25 mg/mL concentration, but the enriched fraction of sugar esters at the highest concentration produced an antifeedant effect of 78% (Table 1).

Table 1. Antifeedant index (%) shown by *C. brevis* on filter paper treated with different *Solanum* spp. extracts.

Extract			Extract concentration	Plant species
Water-ethanol	Dichlomethane-acetone*	Dichloromethane		
9.92 % Ba	32.46 % Ab	31.67 % Aa	2.50 mg/mL	*S. bistellatum*
-6.9 % Ba	52.80 % Aa	39.81 % Aa	12.50 mg/mL	
-1.4 % Ba	57.46 % Aa	43.29 % Aa	25.00 mg/mL	
-7.0 % Bb	16.23 % Ab	10.79 % Ab	2.50 mg/mL	*S. sisymbrifolium*
9.96 % Ba	63.85 % Aa	86.91 % Aa	12.50 mg/mL	
8.66 % Ba	78.20 % Aa	100 % Aa	25.00 mg/mL	

* Enriched fraction of sugar esters

Mean of five replicates (n=30 termites per replicate).

Values followed by the same letter,in lines(capital letters) for each plant extract and concentration in columns (lower case letters), are not different from each other (Tukey's test, P<0.05).

The effects of Solanaceae plant extracts on *Brevicoryne brassicae* (cabbage aphid) were assessed by Lovatto et al. (2004), who reported *S. sisymbrifolium* as the only species with significant repellent activity with a 5% flower extractbeing the most effective. The glandular trichomes present in Solanaceae are related to the plants resistance against herbivore attacks. Investigations of the leaf surface of *Salpichroa origanifolia* (Solanaceae) identified type IV trichomes, while chemical investigations confirmed the presence of acyl sugars with antifungal activity levels similar to those of agrochemicals (Dutra et al., 2008).

The bioactivity of the assayed fractions from the obtained extract from the leaf surface of *S. tuberosum* on the beetle *Leptinotarsa decemlineata* is deterrent or phagostimulant, as reported by Szafranek et al. (2008*)*. The repellency had similar results in both cases. Dichloromethane extracts and their acetone soluble fraction of the two *Solanum* species evaluated in the present study showed repellency against *C. brevis*, particularly at their highest evaluated concentration (Table 2). *Solanum sisymbrifolium* was the most active of the dichloromethane extracts at low concentration.

The dichloromethane-acetone extract for sugars ester from *S. sisymbrifolium* showed an increased repellent effect on *C. brevis*. The same extracts of *S. bistellatum* and *S. sisymbrifolium* showed a repellent effect >90% at 2.5 mg/mL.The *Solanum bistellatum* dichloromethane-acetone extract contained a 50% acyl sugar compounds and the *S. sisymbrifolium* extract contained up to 70% of acylsugars and showed high repellent activity.

Table 2. Mean repellency (%) of *Solanum spp.* extracts on *C. brevis.*

Extract			Extract concentration	Plant species
Water-ethanol	Dichlomethane-acetone*	Dichloromethane		
43.81**Ac**± 15.06	28.73**Bc**± 13.07	50.70**Ab**± 12.10	0.25 mg/mL	*S. bistellatum*
56.09**Ab**± 7.28	41.90**Ab** ± 17.20	42.70**Ab**± 13.69	1.00 mg/mL	
86.32**Aa**± 7.28	92.14**Aa**± 5.49	93.20**Aa**± 4.12	2.50 mg/mL	
32.74**Bb**± 6.60	84.53**Ab**± 2.68	79.91**Ab**± 3.09	0.25 mg/mL	*S. sisymbrifolium*
37.31**Bb**±6.68	90.06**Aa**± 2.01	92.21**Aa**± 2.08	1.00 mg/mL	
50.73**Ca**± 7.02	91.91**Ba**± 1.98	82.67**Ab**± 3.18	2.50 mg/mL	

*Dichloromethane-acetone extract for sugar esters.

Mean of five replicates (n=30 termites per replicate).

Values followed by the same letter in each column, for each plant, are not different from each other (Tukey's test, P<0.05).

The dichloromethane extracts contained waxy compounds embedded in the cuticle at the plant surface where they form a hydrophobic barrier that, protects leaves against dehydration, attack by insects, and diseases (Cesio, 2004; Smith, 1989; Garcia et al., 1995). Nevertheless, the most non-polar hydrophobic compounds in the epicuticular wax like hydrocarbons and wax esters have only dehydration protection capacity and the compounds with anti-insect and antifungal properties are medium polarity compounds (Garcia et al., 1995), which were obtained after partitioning the residue with acetone and freezing it overnight. The chromatographic analysis of the resulting acetone extracts confirmed the absence of the less polar hydrocarbons, wax esters, and n-alkanols and a higher proportion of sugar esters (50% for *S. bistellatum* and 75% for *S. sisymbrifolium*), which are compounds released by glandular type IV trichomes found in plants of the *Solanum* genus.

In order to isolate the bioactive compounds, the sugar ester fraction of both *Solanum* species was fractionated by preparative column chromatography. The main compound in each of them was isolated, in order to characterize them spectroscopically. NMR analyses (^{13}C, ^{1}H and HSQC and TOCSY-HSQC), performed on the purified fractions of *S. sisymbrifolium*, confirmed the structure reported by Cesio et al. (2006): a glycoside of β-hydroxypalmitic with an arabinoxilane. For the compounds isolated from *S. bistellatum,* the same set of NMR experiments were performed allowing the identification of a similar structural pattern as those isolated from *S. sisymbrifolium*. The same glycoside of β-hydroxypalmitic acid and β-xylopyranosil(1-5)-α-furoarabinose template was esterified with two molecules of β-hydroxypalmitic acid and palmitic acid in *S. bistellatum*, as deduced from the GCMS analysis and integration of the diasterostopic hydrogens attached to the α carbon to the carboxyls that appeared in the ^{1}H NMR spectra between δ=2.0-3.0ppm (Fig 1).

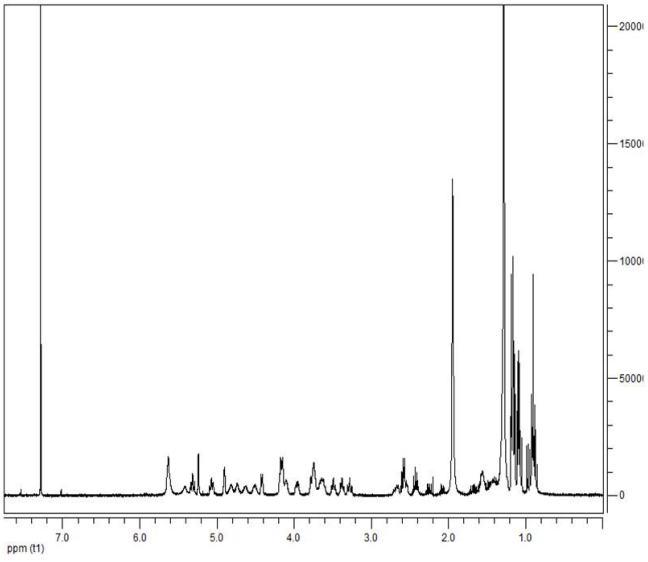

Fig 1. ^{1}H nuclear magnetic resonance of the major sugar ester compound isolated from *S. bistellatum.*

The HSQC-TOCSY distinguished three spin systems, identifying the sugar template: one for the arabinose in furopyranose form, (δ(^{13}C) = 109.3ppm, δ (^{1}H)= 5.23 ppm,) (1, 2 and 3) the second one belonging to xylose in glycopyranose form (δ (^{13}C)= 104.0ppm, δ (^{1}H) = 4.40ppm) and the third one to the hidroxy acids (δ(^{13}C)= 71.40, 78.40ppm, δ (^{1}H) = 4.10ppm).

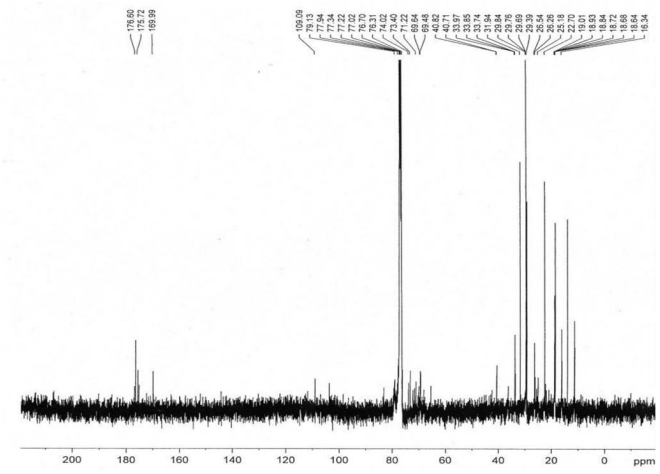

Fig 2. ^{13}C nuclear magnetic resonance of the major sugar ester compound isolated from *S. bistellatum.*

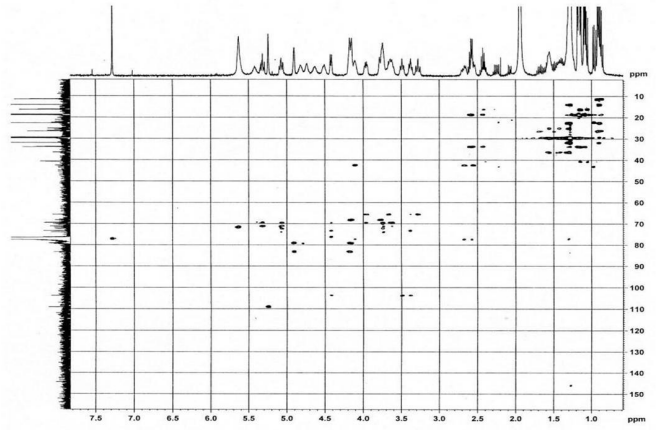

Fig 3. HSQC-TOCSY NMR of the major sugar ester compound isolated from *S. bistellatum.*

The HMBC experiment was useful to identify the glycosidic unions that were the same reported previously. Nevertheless, we were not able to identify the position where the fatty acids were attached. The proposed structure is depicted in Fig 4.

According to the structural data, the most important acyl sugar fraction in *S. bistellatum* has similar structural features as the acyl sugars from *S. sisymbrifolium* (Cesio, 2004): a disaccharide glicosidated with a β-hydroxypalmitic acid. The disaccharide portion of the molecule is also diesterified by two fatty acid residues.

To develop termite control strategies is not straightforward. Once termites colonize the wood, they are not accessible to surface treatments. One alternative is to use preventive and repelling products applied to the wood surface before structural use. Antifeedants are an example of possible surface treatments which could be used to inhibit the insects' ability to penetrate wood. Antifeedant treatments for termite control can be a useful tool for protecting wood as well (Gutiérrez et al., 1999; Sbeghen-Loss et al., 2009). The products and extracts described in the present study show repellent activities that can possibly protect wood against termite attack. Natural products are easily degraded in the environment by many microorganisms (Zhou et al., 2013; You et al., 2014). Particularly, sugar esters are very simple compounds consisting of simple sugars esterified by fatty acids. These properties should be tested in field trials in order to evaluate the persistence of the natural active compound in the environment or design more stable analogs.

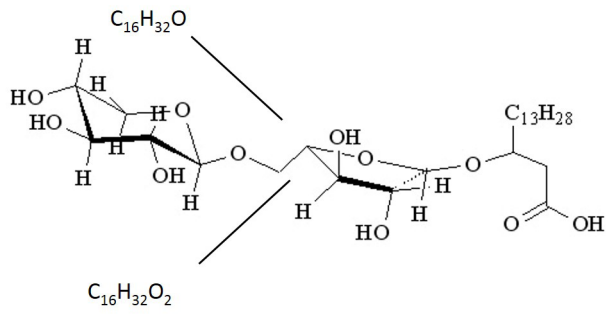

Fig 4. Structure for the major active compound from *S. bistellatum.*

As stated above, a possible strategy to protect wood against termites is to prevent wood colonization by repelling termites from surface entry. In this context, the present work contributes with new tools in that regard by reporting the chemical composition of type IV trichomes exudates from little known Solanaceae spp. which show signficant properties as antifeedants and repellents against the drywood termite, *C. brevis*. The extracts and sugar esters isolated and described in this study show very interesting repellent activities which may be used in pest management.

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