Effects of Usnic, Barbatic and Fumarprotocetraric acids on Survival of *Nasutitermes corniger* (Isoptera: Termitidae: Nasutitermitinae)

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**Abstract**

Lichens (Algal-Fungal association) synthesize unique chemical substances with different biological activities. Three pure lichen compounds were assayed to evaluate their potential insecticidal activity against the termite *Nasutitermes corniger* on Petri dishes. Usnic, fumarprotocetraric and barbatic acids were isolated and purified from the lichens *Cladonia substellata*, *C. verticillaris* and *Cladia aggregata*, respectively, using thin-layer and high-performance liquid chromatography for attesting their purity. Nuclear proton magnetic resonance and infrared spectrophotometry was used for their chemical characterization. After exposure, mortality of termites (workers and soldiers) was determined during 11 days period. The termiticidal effect was influenced by the exposure time and the type of member colony. The results showed that lichen substances, tested at 5, 7 and 10 mg mL⁻¹, have a termiticidal activity (~100%) on worker termites after eight days of treatment, in comparison with controls. However, no significant effect on soldiers was found. These findings indicate that usnic, fumarprotocetraric and barbatic acids are potential compounds for use in the control of this urban pest.

**Abbreviations**: Usnic acid (USN), barbatic acid (BAR) and fumarprotocetraric acid (FUM).

**Introduction**

Termites are eusocial insects that live in large colonies, in which the work is well divided, and all tasks are performed by distinct groups of individuals organized into castes of wingless or winged termites. *Nasutitermes corniger* (Motschulsky), a member of the termite family Termitidae, is one of the most dominant and broadly distributed species of the genus *Nasutitermes*. It is a neotropical species found in roughly 42 Caribbean islands and other countries spanning a longitudinal distance of 6000 km to include parts of Central and South America, in Brazil it is found in the semi-arid region (Scheffrahn et al., 2005). *N. corniger* is highly adaptable to colonization of contrasting habitats in urban, agricultural, and natural environments. Usually termites are evolutionarily and ecologically very successful insects, which preferentially build their nests in trees, soil, roofs and even furniture, feeding on cellulose or lignocellulose digested by symbiotic intestinal microbiota (Breznak et al., 1982). Living in complex societies and being able to digest wood with the aid of a diverse symbiotic gut fauna, seems to be the basis for this success story.

These arthropods have considerable ecological importance as primary consumers, assisting in the decomposition process and recycling of nutrients from plants, and also as nitrogen-fixing organisms. They are responsible for the distribution of important nutrients in the soil, and can be used as a bioindicator...
of soil quality and fertility. However, in urban areas some species are considered pests due to the destruction caused to buildings and the deterioration of paintings, books, old documents and monuments of historical importance (Verma et al., 2009). One estimate from 2005 put the annual damage caused by termites at about US$ 50 billion worldwide, with the US alone investing more than US$ 11 billion in termite control in 1994 (Korb, 2007). In Brazil, termite damage affected 42.7% of constructions in 2002, with losses of about 3.5 billion dollars in the past 20 years in the state of São Paulo (Milano & Fontes, 2002).

The usual method for controlling termites involves the use of synthetic compounds. However, insecticides, such as organophosphates and pyrethroids, are characterized by their extremely high toxicity against ecosystems and their biota. Thus, research has been carried out on natural products from plants (flavonoids, alkaloids, terpenoids and tannins) (Santana et al., 2010) and lichens (lectins, depsides and depsidones) (Cetin et al., 2008), due to their biological activities, including anti-herbivory and insecticidal properties.

Lichens are the result of a symbiotic relationship between fungi and algae and/or cyanobacteria in a process denominated lichenization. The characteristics that make lichens different from other fungi are the formation of an air-exposed thallus, slow growth and the longevity of the fruiting bodies (Sipman & Aproot, 2001). Lichens are complex biological structures with unique characteristics, such as the production of distinctive substances grouped in four well differentiated chemically classes (depsides, depsidones, dibenzofurans and depsones), named as lichen acids (Howell et al., 2003; Eisenreich et al., 2011). Those compounds are phenolic acid derivatives with free hydroxyl groups, which can be toxic to most other living beings. The interest in the properties of lichen substances goes as far back as the 17th century. According to Solhaug et al. (2009), the deterrent action against herbivory is the most likely ecological function of such substances. Several studies have reported herbicidal, anti-herbivory and insecticidal activity of different lichen metabolites (Lawrey, 1980; Reutimann & Scheidegger, 1987; Giez et al., 1994; Rundel, 1978; Nimis & Skert, 2006; Asplund et al., 2009; Silva et al., 2009; Pöykkö et al., 2010). However, the effectiveness of most lichen acids against higher termites (Termitidae) has not been evaluated. The aim of the present study was to evaluate the insecticidal potential of phenolic compounds: usnic acid (USN), barbatic acid (BAR) and fumarprotocetraric acid (FUM), extracted and purified from the lichens: Cladonia substellata, C. verticillaris and Cladia aggregata, respectively, collected in northeastern Brazil against N. corniger, a termite of ecological importance that is well adapted to urban areas.

Materials and Methods

Harvesting and storage of lichens

C. substellata Vainio was collected in the municipality of Mamanguape (Paraíba, Brazil), C. verticillaris (Raddi) Fr. was collected in the municipality of Alhandra (Paraíba, Brazil) and C. aggregata (Sw.) Nyl. was collected in the municipality of Bonito (Pernambuco, Brazil). About 150 g of each species were harvested in the selected areas. Thalli were dried in air flow and stored in the dark at room temperature (28 ± 3 °C). A sample of each species was deposited in the Herbário-UFP-Geraldo Mariz, Universidade Federal de Pernambuco (Brazil), whose register numbers are 34402 (C. substellata), 36431 (C. aggregata) and 361638 (C. verticillaris).

Acquisition of organic extracts

The thallus sample from C. substellata (100 g) was washed, ground and submitted to extraction with diethyl ether (Darmstadt, Germany) in a Soxhlet extractor at 40 °C for 16 h. The extract was filtered through Whatman filter paper (No. 1) and evaporated in a rotary evaporator (Buchler Instruments, Fort Lee, NJ, USA) in a water bath at 40 °C until dry. The same procedure was performed for the thallus samples of C. aggregata (100 g). The ether extracts of C. substellata and C. aggregata were used for the isolation and purification of USN and BAR, respectively. The thallus sample from C. verticillaris (100 g) was washed, ground and submitted to acetone extraction (Merk®, Darmstadt, Germany), following the same procedures used for C. substellata and C. aggregata. The acetone extract of C. verticillaris was used for the purification of FUM (Asahina & Shibata, 1954).

Lichen acids: isolation and purification

Usnic acid (USN)

USN was obtained from the ether extract of C. substellata. Isolation and purification were performed using a classic chromatographic procedure in a silica gel column (porosity 70 to 230 mesh). The mobile phase was chloroform: n-hexane (80:20 v/v) (Odabasoglu et al., 2006).

Barbatic acid (BAR)

The ether extract of C. aggregata was successively washed with chloroform in a G4 funnel under pressure to obtain pure crystals of BAR. Additionally details related to the extraction and purification of BAR are provided by Martins et al. (2010).

FUM

The acetone extract of C. verticillaris was successively treated with cold methanol to obtain FUM crystals as described Gudjnsdottir and Ingolfsdottir (1997).

Identification of extracted phenolic compounds, purified substances, confirmation of molecular structure

Identification and degree of purity (> 95%) were evaluated using thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC). Confirmation of the molecular structure was performed with proton nuclear magnetic resonance (1H NMR) and infrared (IR) spectroscopy. The USN standard was obtained from Merk®
Darmstadt, Germany, and BAR and FUM were obtained from the Natural Products Laboratory of the Universidade Federal de Pernambuco, Brazil.

**Thin-layer chromatography (TLC)**

Samples of 0.1 mg of the purified compounds, organic extracts and phenolic standards were dissolved in acetone (0.5 mL). Next, 1 µL of the solution was applied to a silica gel plate (Gel 60 F<sub>254</sub> Merk® Darmstadt, Germany) measuring 20 x 20 cm. TLC assays were performed under ascending conditions using the solvent systems A (toluene/dioxane/acetic acid, 36:9:1, v/v/v) for USN and BAR and B (n-hexane/diethyl ether/formic acid, 6.5:4:1 v/v/v) for FUM. Bands were observed under short (256 nm) and long (366 nm) ultraviolet (UV) light and visualized using a sulfuric acid solution (10%) and heating to 50 °C for 20 minutes to promote the color reaction. The phenolic composition was evaluated by determining R<sub>f</sub> values and comparisons with those obtained for standard USN, BAR and FUM (Culberson, 1972).

**High performance liquid chromatography (HPLC)**

The same material analyzed by TLC was submitted to HPLC assays as described Legaz and Vicente (1983), in a Hitachi Chromatograph (655 A-11, Tokyo, Japan) coupled to a CG437-B UV detector set at 254 nm. For the separation, a C-18 reverse phase column MicroPack MCH-18 de 300 mm × 4 mm, Berlin, Germany (Merk® KGaA, Darmstadt, Germany) was used. The samples were injected at a concentration of 0.1 mg mL<sup>-1</sup> and were developed in an isocratic mode using as mobile phase methanol/deionized water/acetic acid, 80:19.5:0.5 (v/v). Other analytical parameters were: volume of injection 20 µL, attenuation 0.16, pressure 87 atm, flow rate 1.0 ml min<sup>-1</sup> at room temperature (28°C ± 3°C). The results were analyzed by comparing the retention time (R<sub>t</sub>) of substance in the column, and peak area, taking the standards as reference.

Proton nuclear magnetic resonance (¹H NMR) and infrared (IR) spectroscopy

The chemical structures of the isolated compounds (USN, BAR and FUM) were confirmed using MNR<sup>¹H</sup> and IR spectroscopy. The MNR<sup>¹H</sup> data were obtained from a Varian Unity Plus 300 MHz spectrometer using DMSO-d<sub>4</sub> as solvent in 5 mm tubes at room temperature. IR analyses were performed in a Bruker Fourier spectrometer (model IFS 66) with KBr disks.

**Termiticidal assay**

A colony of *N. corniger* termites was collected from the campus of the Universidade Federal de Pernambuco (Brazil) and the termites were identified by Dra. Auristela C. Albuquerque (UFPE). Termitic colony was maintained in the vegetation house at the Department of Plant Biology (UFPE). Termiticidal activity was performed using a method based on Kang et al. (1990). Filter paper disks (4 cm in diameter) were soaked with solutions (200 µL) of the USN, BAR and FUM at concentrations of 5, 7 and 10 mg mL<sup>-1</sup> solubilized with acetone. Each experiment unit consisted of a Petri plate with the deep plate covered by filter paper disk. The disks were dried at 28 °C and placed in Petri dishes (90 x 15 mm). A total of 20 termites (workers and soldiers, in the proportion of 4:1 respectively) were carefully transferred to each plate and kept in the dark at 27 ± 1 °C with 80% relative humidity. Evaluation of percentage insect survival was made daily until 11-day period. Bioassay was achieved in quintuplicate and survival rates were obtained for each treatment and expressed as mean ± SD.

**Statistical analysis**

The statistical analyses were performed with the Proc Lifetest of the SAS program (SAS Institute, 2001), using probit analysis with 95% confidence intervals. Significant differences were determined using the Student’s t-test (p < 0.05).

**Results**

**Chemical characterization and identification of lichen compounds**

Diagnostic secondary metabolites for the three lichens species are well known with Rf values and spot characteristics. In the thin layer chromatogram (TLC) of *C. verticillaris* acetone extract spot with Rf 0.18 was shown, TLC data demonstrate the presence of FUM as the major phenolic compound in this extract. Traces of two other compounds were also found, with R<sub>f</sub> of 0.08 and 0.19. The same result was observed for ether extracts of *C. substellata* and *C. aggregata*. Spot with R<sub>f</sub> 0.50 demonstrates the presence of USN the major phenolic substance. Data were in agreement with the purified standard acids (90 x 15 mm). A total of 20 termites (workers and soldiers, in the proportion of 4:1 respectively) were carefully transferred to each plate and kept in the dark at 27 ± 1 °C with 80% relative humidity. Evaluation of percentage insect survival was made daily until 11-day period. Bioassay was achieved in quintuplicate and survival rates were obtained for each treatment and expressed as mean ± SD.

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The chemical structures of the isolated compounds from *C. verticillaris*, *C. substellata* and *C. aggregata* (Figures 3A, B and C) were confirmed by the determination of the melt point and through H<sup>1</sup>-NMR and IR spectroscopy. The data are described as follows: **FUM**: white solid, MP. 252-260°C (decomposition), ¹H NMR (300 MHz, DMSO-d<sub>6</sub>) δ<sup>H</sup> (H; mult.; int.): 2.38 (3H; s; CH<sub>3</sub>-9), 2.41 (3H; s; CH<sub>3</sub>-9). 5.26 (2H; s; CH<sub>2</sub>-8'), 6.60 (2H; s; CH=2'; CH=3'), 6.80 (1H; s; CH=5), 10.53 (1H; s; CH=8), 11.93 (1H; s; C-4-OH or C-2'-OH). IR. (KBr): 3490, 3095, 2950, 2851, 1745, 1701, 1654, 1329, 1259, 1229, 1209, 1156, 1105 cm<sup>-1</sup>; USN: yellow solid crystal, MP, 201-203°C, [α]<sub>D</sub> +25 °C + 493 °C (CHCl<sub>3</sub> c, 1.00 ¹H NMR (300 MHz, DMSO-d<sub>6</sub>) δ<sup>H</sup> (H; mult.; int.): 1.73 (3H; s; CH<sub>3</sub>-13), 2.08 (3H; s; CH<sub>3</sub>-16), 2.64 (3H; s; CH<sub>3</sub>-15), 2.65 (3H; s; CH<sub>3</sub>-18), 5.92 (1H; s; C-4-H), 11.04 (1H; s; C-10-OH), 81
13.29 (1H; s, C-8-OH), 18.89 (1H; s, C-3-OH). IR. (KBr): 3090, 3007, 2930, 1692, 1632, 1560, 1454, 1370, 1357, 1340, 1330, 1289, 1230, 1190, 1143, 1118, 1070, 1039, 959, 940, 840, 818, 700 cm\(^{-1}\); \textbf{BAR}: gray solid crystal, MP, 188-189\(^\circ\)C, \(\textsuperscript{1}H\) NMR (300 MHz, DMSO-d\(_6\)): 1.99 (3H; s; CH-8'), 2.00 (3H; s; CH\(_3\)-8), 2.47 (3H; s; CH-9'), 2.56 (3H; s; CH\(_3\)-9), 3.86 (3H; s; CH3-O-4), 6.60 (1H; s; CH-5), 6.69 (1H; s; CH-5'), 10.73 (1H; s; C-2-OH). IR. (KBr): 3092, 2992, 2858, 1659, 1631, 1399, 1290, 1233, 1154, 1080 cm\(^{-1}\).

The chemical analysis revealed a total absence of other lichen acids and purity of lichen acids, employed to termiticidal assay, was confirmed.

The insecticidal potential of the purified USN, FUM and BAR was evaluated on \textit{N. corniger}. Three concentrations were assayed (5, 7 and 10 mg mL\(^{-1}\)) and the percentage of survival was evaluated over an 11-day period. Survival of \textit{N. corniger} incubated in the absence (control treatment) or in the presence of each phenolic are shown in Tables 1, 2 and 3.
Table 1. Percentage of termite worker survival following treatment with usnic (USN), fumarprotocetraric (FUM) and barbatic (BAR) acids at concentration of 5 mg mL\(^{-1}\). Symbol (-) indicates 0% survival. Treatments were compared with the control and among treatments. Asterisk (*) indicates a significance difference at p< 0.05.

<table>
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<tr>
<th>5 mg mL(^{-1})</th>
<th>Survival (%)</th>
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<tr>
<td>Day</td>
<td>0</td>
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<tr>
<td>Control</td>
<td>100±0</td>
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<tr>
<td>USN</td>
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<td>FUM</td>
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<td>BAR</td>
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Table 2. Percentage of termite worker survival following treatment with usnic (USN), fumarprotocetraric (FUM) and barbatic (BAR) acids at concentration of 7 mg mL\(^{-1}\). Symbol (-) indicates 0% survival. Treatments were compared with the control and among treatments. Asterisk (*) indicates a significance difference at p< 0.05.

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<th>7 mg mL(^{-1})</th>
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<tr>
<td>Day</td>
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<td>Control</td>
<td>100±0</td>
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<tr>
<td>USN</td>
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<td>FUM</td>
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<td>BAR</td>
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Table 3. Percentage of termite worker survival following treatment with usnic (USN), fumarprotocetraric (FUM) and barbatic (BAR) acids at concentration of 10 mg mL\(^{-1}\). Symbol (-) indicates 0% survival. Treatments were compared with the control and among treatments. Asterisk (*) indicates a significance difference at p< 0.05.

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<th>10 mg mL(^{-1})</th>
<th>Survival (%)</th>
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<td>Day</td>
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<td>Control</td>
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<td>USN</td>
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<td>BAR</td>
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Discussion

Describing the phenolic composition of *C. verticillaris* occurring in northeastern Brazil, Ahit et al. (1993) identified the presence of the FUM, protocetraric acid, orcinol, methyl-orcinol carboxylate and atranorin. For *C. substellata* and *C. aggregata*, the same authors describe the presence of the compounds identified in this study, namely, USN in *C. substellata* and BAR in *C. aggregata*, which are dominant chemotype. However, the authors also found stictic, constictic, cryptostictic, norstictic and connortistic acids in the *C. substellata* from NE of Brazil. For *C. aggregata* the chemotype with the stictic acid group is also known. This variety of chemotypes was confirmed in the extracts TLC analyses, where traces of other compounds were also found (Figure 1). In the present study, we tested dominant chemotypes to improve their amount of purification, but the other compounds as stictic and norstictic acids has also been described as biocative compounds (de Paz et al., 2010; Pejin et al., 2013).

Studies on the termiticidal activity of lichen substances are scarce. However, previous investigations have demonstrated that arthropods, such as moths, mites and beetles, avoid feeding on species of lichens that produce FUM, stictic and pulvinic acids. These substances are described as toxic compounds with a bitter taste (Lawrey, 1987; Reutimann & Scheidegger, 1987). According to Nimis and Skert (2006), there is a close relationship between the presence of the lichen metabolites and grazing phenomena. The observations of the cited authors indicate that species that contain atranorin, calcium oxalate, gyrophoric acid, lecanoric acid, USN and zeorin have an important ecological function by either repelling or attracting insects, depending on the compounds found in the thallus. Based on this, some studies demonstrated that the toxicity of extracts isolated from lichen samples against pests was related to their components (Emmerich et al., 1993; Balaji et al., 2007; Cetin et al., 2008). Extracts isolated from different lichen species might have different toxicity levels, which can be attributed to their different chemical composition (Sahip et al. 2008; Yildirim et al. 2012; Emsen et al. 2015). Therefore, lichens can be potential sources of natural compounds for pest management.

Natural products are now being considered as alternatives to the arsenal of synthetic compounds. The insecticidal activity of natural products is of considerable interest to researchers, as these compounds may be the basis for strong new insecticides, offering an alternative to extremely toxic synthetic insecticides.

Termites consume many types of food such as cellulolic materials, for example paper and wood. The evaluation of lichens acids effect on *N. corniger* was performed using filter paper soaked with the purified lichen compounds. The results showed that the survival of termite workers treated with lichen substances was significantly reduced in relation to the control group within a short period of time. Moreover, the insecticidal efficiency of these substances may be extended to other types of insects. For example, assays involving *Spodoptera* larvae submitted to a diet with oxyphysodic (11.2 µmol g⁻¹ wt) and FUM (30.5 µmol g⁻¹ wt) acids or calcin (37.6 µmol g⁻¹ wt) obtained from lichens demonstrated a strong increase in the larval period and a high incidence of malformations, indicating a prolonged effect of the lichen substances tested (Giez et al., 1994).

Depsides, such as BAR, lecanoric acid, rosolic acid and orcein, and depsidones, such as FUM, protocetraric and stictic acids derived from orcinol, are found in lichens, particularly those of the genera *Cladonia* and *Cladia*. The insecticidal activity of these substances has also been described. For example, the depside lecanoric acid and the depsidone physodic acid are lethal to larvae of the caterpillars *Helicoverpa armigera* and *Cleorodes lichenaria*, respectively (Balaji et al., 2007; Pöykkö et al. 2010). In this study, it has been demonstrated that BAR and FUM are lethal to worker termites at 5, 7 and 10 mg ml⁻¹ concentrations after 7 days of treatment (Tables 1, 2 and 3). Dibenzoferun USN, which is a major compound in *C. substellata*, caused 100% mortality of termite workers at a concentration of 10 mg ml⁻¹ after 7 days of treatment (Table 3). This compound has demonstrated considerable efficacy against several genera of insects of interest to public health. Cetin et al. (2008) describe the action of USN against *Culex pipiens* under laboratory conditions, demonstrating 100% lethality in day 3 and 4 instar larvae at concentrations of 5 and 10 ppm, with LC₅₀ at 0.8 ppm. The data indicate that USN may be used as a new bioinsecticide and its chemical structure can be used as a prototype for stronger insecticidal compounds.

Besides the phenolic derivatives (depsides and depsidones) from the secondary metabolism, the lichen thallus is rich in substances from its primary metabolism, such as complex carbohydrates and lectins. Lectins have an important function in the symbiotic association with fungi and algae (Diaz et al., 2016) and have proven to be effective against *N. corniger*. Lectin (ClaveLL) extracted and purified from *C. verticillaris* exhibits termiticidal activity, inducing the death of both workers (LC₅₀: 0.196 mg ml⁻¹) and soldiers (LC₅₀: 0.5 mg ml⁻¹) after 10 days of treatment (Silva et al., 2009). The results obtained in the present study showed that FUM purified from *C. verticillaris* reduced the survival of termite workers at all concentrations tested. These findings, supported by Silva et al. (2009), justify *C. verticillaris* lectin to be a potential bioinsecticide.

Termites have the ability to digest cellulose due to the presence of endoglucanases, exoglucanases and β-glucosidases with endogenous and/or symbiotic origin in their gut (Breznak & Brune, 1994). According to Yamaoka (1996), cellulose digestion in the intestine of termites occurs with the assistance of symbiont protozoa or more types of bacteria present therein. Zhou et al. (2008) tested efficacy of three prototype termite cellulase inhibitors as novel termite control agents. Lichen substances have many biological activities described.
by different authors. For example, bactericidal effects have been found for BAR on *Staphylococcus aureus*, FUM on *Klebsiella pneumoniae* and USN on *Mycobacterium tuberculosis* (Martins et al., 2010, Neeraj & Behera, 2011; Lucarini et al., 2012). It is quite possible that the effect of such chemical substances is associated with their antibacterial activity or their acidic nature and bitter taste, which reduces the palatability of the thallus (Ivanova & Ivanov, 2009).

Moreover, lichens produce complex carbohydrates, such as evernin, inulin, lichenin and isolichenin, which cause irritation of the intestinal tract and hinder the digestion of many substances (Lawrey, 1987). The precise action mechanism of lichen compounds as toxic molecules is still unknown.

Termites are ecologically beneficial, however biodeterioration of cellulose-based materials by termites is a serious problem and are considered as plague-insects. The control of these insects is currently based on the use of chemical insecticides, which can contaminate the environment, are toxic to humans and animals and can lead to the emergence of resistant insects. Thus, new effective methods for termite control have been searched.

FUM, BAR and USN exhibited termiticidal effect for worker caste of *N. corniger*, indicating their potential as bioinsecticides. This way, our findings offers an opportunity to develop alternative strategies to control this pest. Further studies are needed for discovering new potential termiticidal compounds produced by lichens and characterization of their action mechanisms against pests.

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References


