Correlation between bioaccumulated phytocompounds and bioactivities in *Nasutitermes macrocephalus* (Silvestri, 1903) (Isoptera: Termitidae) and its nest extracts against multi-resistant bacterial strains

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Received 28 July 2016, revised 24 January 2017

Termites are among the species most commonly used in folk medicine in various locations worldwide. Of these, *Nasutitermes macrocephalus* is often used in the treatment of various diseases that affect humans, however, with no supporting scientific evidence. From this perspective, this study aimed to evaluate the antibacterial activity and modulating effect of bacterial resistance of *N. macrocephalus* and its nest extract. The minimum inhibitory concentration (MIC) of antimicrobial agents used in clinical practice is determined in the presence and absence of the ethanol extract of *N. macrocephalus* and its nest in a subinhibitory concentration (125 µg/mL) against multi-resistant strains of *Escherichia coli* and *Staphylococcus aureus*. The extract showed no significant antimicrobial activity (MIC > 1000 µg/mL). However, there was a considerable reduction in MIC of antimicrobials when combined with the extract, especially erythromycin, ampicillin and gentamicin. The results indicate that *N. macrocephalus* and its nest are a promising source of substances that can be used in combating multi-drug-resistant bacteria. Additional studies are needed to identify the active components responsible for such activity.

Keywords: Termites, Ethnozoology, Natural products, Bacterial resistance, Modulatory activity

IPC Int. Cl. 8: A01N, A61K, C12N, C12M, C07, C08

Insects, a group of animals more numerous and diverse than any other, have played an important role in various parts of the world as sources of medicinal resources and food, as aesthetic-decorative inspiration, and in the religious and magical practices of different ethnic groups1. Whole insects and other arthropods, as well as substances extracted from them, have been used worldwide as medicinal resources by human cultures2. For a variety of reasons, insects and their chemical defense systems represent a valuable source of novel chemistry that certainly merits further investigation as a source of new medicinal compounds as well as other applications3. Termites (Isoptera) are among the insect species most frequently used in traditional folk medicines around the world4. In Brazilian semi-arid region various species of termites have been used in folk medicine by the local population2, including *Nasutitermes macrocephalus*, which is employed in the treatment of various diseases and conditions, such as influenza, asthma, bronchitis, coughs, sinusitis, tonsillitis and hoarseness5. This species is commonly found in South America, mainly in tropical forests and ecosystems of semi-arid regions6,7. *N. macrocephalus* builds arboreal nests, with a volume that may exceed 1000 liters8. In tropical forests, the population of these nests may vary from 150.000 to 1.500.000 individuals, with 15 to 544 individuals/m29. It is a keen consumer of wood at various stages of decomposition10. The Caatinga, predominant ecosystem of the Brazilian semiarid region, presents a large number of plants and animals that have been used by the local population for medicinal purposes, thus suggesting a great potential from the perspective of bioprospecting10. Therefore, studies to analyze the pharmacological potential of animals and plants of the Caatinga may contribute to

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ensuring that local natural resources are properly valued, not only ecologically, but also economically and socially. In this context, pharmacological and biochemical investigations to identify the true efficacy of medicines produced from termites are essential, since its use is widespread in traditional medicine for the treatment of various diseases and conditions in the Brazilian semi-arid region. Therefore, this study aimed at evaluating, through experimental models in vitro, the extract of N. macrocephalus and its nest as an antibacterial agent and as a modifier of resistance against pathogenic microorganisms.

Methodology

Zoological material

The collection of N. macrocephalus along with the nest was performed on the farm Farinha, municipality of Pocinhos, semiarid region of Paraíba state, (7°07´S, 36°07´W). The species was identified by Prof. Alexandre Vasconcellos from the Department of Systematics and Ecology at the Federal University of Paraíba (UFPB). A sample was deposited to the Isoptera collection at the Center of Exact and Natural Sciences UFPB under the number CICB 68.

Extract preparation

Twenty gram of termites with nest was subjected to extraction by maceration with 100 mL of absolute ethanol for 5 days at room temperature (25±3 °C) with occasional stirring. After filtration, the extracts were concentrated on a rotary evaporator at 40 °C. Before microbiological testing the extract was diluted into 10 % Dimethylsulphoxide (DMSO) solution.

Phytochemical tests

Determination of total polyphenols

The total polyphenol content of plant extracts was measured using spectrophotometry in the visible region by the method of Folin-Ciocalteu described by Chandra and Mejia with minor modifications. The extracts (25 mg) were dissolved in distilled water to obtain a final concentration 200 µg/mL. From each solution, a 1 mL aliquot was added to 1 mL of 1 mol/L Folin-Ciocalteu reagent. This mixture remained undisturbed for 2 min before the addition of 2 mL of 20 % (w/v) Na2CO3 solution and left undisturbed for 10 min. Thereafter the reading was performed Spectrophotometer Shimadzu, model UV-mini 1240, at 757 nm. The calibration curve was obtained with gallic acid at concentrations between 1 and 40 µg/mL.

Determination of total flavonoids

The total flavonoids were determined by the method described by Meda et al. The extracts were diluted with methanol at 1000 µg/mL. To the 5 mL of each test solution was added the same volume of 2 % (w/v) AlCl3 solution in methanol. This mixture remained undisturbed for 10 min before the UV spectrophotometric reading at 415 nm wavelength. The total flavonoids were determined by the calibration curve using quercetin (Sigma-Aldrich) as standard at concentrations between 2 and 30 µg/mL.

Determination of condensed tannins

The content of condensed tannins was verified through the method of vanillin-HCl described by Makkar & Becker, where 0.25 mL of the sample were added to 1.5 mL of vanillin solution in methanol (4 % w/v) and subsequently, 0.75 mL of concentrated HCl (37 %). After HCl addition, the tube content was shaken in water bath at 30 °C for 3-4 sec before reading on a spectrophotometer at 500 nm wavelength. Catechin was used as standard.

Determination of saponins

The quantification of total saponins followed the method described by Makkar et al. 250 µL of 8 % vanillin solution in ethanol was added to 250 µL extract solution in 80 % methanol; then 2.5 mL of 72 % sulfuric acid were added. The tubes were incubated at 60 °C in a water bath for 10 min and transferred to an ice bath, staying for 4 min. The absorbance reading at 544 nm was performed against a blank consisting of the vanillin solution, 80 % methanol and sulfuric acid. The calibration curve was obtained from a disogenin solution at concentrations between 100 and 500 µg/mL.

Drugs

All the drugs tested were obtained from Sigma Chemical Corp., St. Louis, MO, USA, and dissolved in sterile water before use.

Minimal inhibitory concentration (MIC) and determination & modulation activity

We used the clinical isolates of Staphylococcus aureus UEPB01, resistant to ampicillin, ciprofloxacin, cephaloxin, erythromycin, penicillin and amoxicillin and Escherichia coli UEPB01, resistant to amoxicillin, cephalothin, levofloxacin, chloramphenicol, tetracycline and gentamicin. The tested bacterial strains were incubated at 37 °C for 24 h in Mueller–Hinton agar and maintained on agar slants in tubes on the same medium. The minimum inhibitory concentration (MIC) was determined by microdilution method in 96-well plates using Mueller-Hinton broth. Colonies of microorganisms were suspended in 0.9 % saline
solution and the suspension adjusted by spectrophotometric method at 625 nm to a final concentration of 5 x 10^6 CFU/mL. Were performed serial dilutions of the extract in the range of 1000 to 3.9 μg/mL and antibiotics in the range of 2500 to 2.4 μg/mL. DMSO 10 % was included as negative control. The plates were incubated at 37 ± 1 °C for 24 h. Bacterial growth was indicated by addition of 20 μL of 0.01 % resazurin aqueous with incubation at 37 °C ± 1 °C for 2 h, and MIC values were identified as the lowest concentration in which no bacterial growth is visible. Evaluation of extracts as modulators of antibiotic resistance was performed according to Coutinho et al.\textsuperscript{15}. The MIC of the antibiotic was determined in presence and absence of sub-inhibitory concentrations (MIC/8). Plates were incubated as described above and each assay was performed in triplicate.

**Statistical analysis of microbiological tests**

The test results were expressed as geometric means. The two-way analysis of variance followed by Bonferroni post-test was applied using the GraphPad Prism 5.0 software.

**Results and discussion**

Through chemical testing it was possible to determine the presence and concentration of substances originating from the plant secondary metabolism. The concentration of these compounds is present in Table 1 and expressed in equivalent milligrams of standards used. In the microbiological testing, there was no significant antimicrobial activity of the extract of *N. macrocephalus* against *S. aureus* UEPB01 and *E. coli* UEPB01 (MIC > 1000 μg/mL). However, the extract when combined with antimicrobials in subinhibitory concentration (125 μg/mL) afforded a significant reduction in the MIC of the antibiotic tested, except for levofloxacin against *E. coli* (Figs. 1&2).

The synergistic effect of the extract tested with antibiotics against *S. aureus* UEPB01 was demonstrated especially when combined with erythromycin, causing a marked reduction in MIC from 2500 to 996 μg/mL. This reduction was also observed in the MIC of ampicillin, 992 to 78 μg/mL (Fig. 1). Against *E. coli* UEPB01 this effect was observed in association with gentamicin extract, reducing the MIC of 156 μg/mL to 39 μg/mL (Fig. 2).

In recent years, there has been observed an increase of bacterial resistance to conventional antibiotics, making the development of new drugs with antibacterial properties necessary in order to address this situation\textsuperscript{16}. From this perspective, various animal species have been methodically tested by researchers as a source of drugs for modern medical science, and the current number of animal sources for the production of basic medicines is extensive\textsuperscript{17}.

Arthropods have attracted attention because of their potential as a source of medically relevant substances, especially the class Insecta\textsuperscript{3}. Among insects, termites are some of the most commonly used in traditional folk medicine in the world\textsuperscript{15,18}. In work of Figueiredo et al.\textsuperscript{19}, a compilation of data was performed where the species *N. macrocephalus* was the most frequently recorded and was found to be widely used as a therapeutic tool in the treatment of asthma, hoarseness, and sinusitis, among other ailments.

The results of this study corroborate the works of Coutinho et al.\textsuperscript{15,17} and Chaves et al.\textsuperscript{20} in which, even without seeing direct antimicrobial activity of *N. corniger* extracts, they found a reduction in the MIC of specific antibiotics to be associated with these extracts. The authors indicated the species *N. corniger* and possibly other species of termites as a source of natural products to modify activity of antibiotics against multi-resistant bacteria.

The synergism observed in this study between the *N. macrocephalus* extract and antibiotics tested may be due to the presence of secondary metabolites present in the extract that are synthesized by plants in response to microbial infections. The chemical analysis carried out in this work revealed the presence of polyphenols, flavonoids, tannins and saponins in the extract. According Cowan\textsuperscript{21}, these metabolites have several activities, including the antimicrobial activity and a previous study with *N. corniger* revealed almost the same concentration of these metabolites\textsuperscript{20} with similar biological results. All these metabolites can affect the lipid bilayer of the bacteria, disrupting the cell membrane and enhancing the influx of antibiotics and consequently their antibiotic effect. *N. macrocephalus* is a xylophage species, and the presence of these secondary metabolites are from the support plant used as a food source; these compounds may be found in the digestive system of termites.

**Table 1 — Concentration of secondary metabolites (mg/g) determined for the extract of *N. macrocephalus* and this nest**

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>Total polyphenols</th>
<th>Total flavonoids</th>
<th>Condensed tannins</th>
<th>Total saponins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration</td>
<td>27.56 ± 4.31</td>
<td>11.75 ± 1.03</td>
<td>12.46 ± 2.14</td>
<td>50.19 ± 19.12</td>
</tr>
</tbody>
</table>

The authors indicated the species *N. corniger* and possibly other species of termites as a source of natural products to modify activity of antibiotics against multi-resistant bacteria.
Evidence of antimicrobial products isolated from these animals has been reported, like the peptides such as spinigerin and termicin isolated from Pseudocanthotermes spiniger, which showed antifungal and antibacterial activity. Studies addressing the molecular biology of the termites Nasutitermes demonstrated their potential as producers of antimicrobial peptides.

In a broader perspective, natural product obtained from various kinds of animals has been demonstrated to have potential to accentuate the action of antibiotics against multiresistant bacteria. Substances extracted from the skin of Rhinella jimi, Ameiva ameiva and Tropidurus hispidus, the body fat of Tupinambis merianae, Gallus gallus domesticus and Spilotes pullatus showed promising results regarding the modification of bacterial resistance. Among the compounds from natural sources, extracts are complex mixtures which make microbial adaptability difficult, and for this reason, their use as an antimicrobial agent lowers the possibility of microorganisms acquiring resistance to its action. When combined with antibiotics, extracts can have direct action against various bacterial species, either increasing or modulating the activity of a particular antibiotic, thereby reversing the natural resistance of bacteria to specific antibiotics.

Traditional significance of study to the society/researchers

This study has a great significance for two different kinds of societies: to the traditional population groups, this study contributes with an accurate demonstration that some drugs have not be used in association with the animal droppings or used to build a nest.

In a broader perspective, natural product obtained from various kinds of animals has been demonstrated to have potential to accentuate the action of antibiotics against multiresistant bacteria. Substances extracted from the skin of Rhinella jimi, Ameiva ameiva and Tropidurus hispidus, the body fat of Tupinambis merianae, Gallus gallus domesticus and Spilotes pullatus showed promising results regarding the modification of bacterial resistance. Among the compounds from natural sources, extracts are complex mixtures which make microbial adaptability difficult, and for this reason, their use as an antimicrobial agent lowers the possibility of microorganisms acquiring resistance to its action. When combined with antibiotics, extracts can have direct action against various bacterial species, either increasing or modulating the activity of a particular antibiotic, thereby reversing the natural resistance of bacteria to specific antibiotics.

Conclusion

The results indicate that the N. macrocephalus extract and its nest have modulatory activity for bacterial resistance against strains resistant to multiple drugs, highlighting the possibility of its use in antimicrobial therapy.

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