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Research Article

Toxicity of *Croton urucurana* Against the Subterranean Termite *Heterotermes sulcatus* (Isoptera: Rhinotermitidae)

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Abstract

The chemical insecticides used in soil against the subterranean termites result in polluting of soil ecosystems and become causes of human health hazards because of such risks, there is a need of developing ecofriendly baits for the control of these termites. With this objective, a study was carried out to evaluate the termiticide potential of ethanol extract of bark; ethanol-water, hexane and ethyl acetate fractions of bark and ethyl acetate fraction of leaf of *Croton urucurana* against the subterranean termite, *Heterotermes sulcatus* (Hagen). The experiments were performed in the laboratory with 100 foraging workers of this termite using no-choice bioassay method. Filter paper treated with crude extract and fractions of *C. urucurana* at concentrations of 250, 500, 1000, 3000, 5000, 8000 and 10,000 ppm were used. A piece of filter paper treated with respective solvent only was used as a control e.g., control 1 (water), control 2 (ethanol solvent). The substitute variables evaluated were daily mortality, lethal concentration LC_{50} and LC_{95} and lethal time LT_{50} and LT_{95}. It was found that the crude extract and ethanol-water fraction resulted in the lowest LC_{50} and LC_{95} values as well as these formulations also presented lowest LT_{50} and LT_{95} values among all the treatments, hence making these formulations most toxic among the tested ones against *H. sulcatus*, *C. urucurana* seemed to have bioactive compounds with termiticide effect and it can be considered as potential alternative to synthetic insecticides for the control of termites.

Key words: Plant extract, secondary metabolites, subterranean termite, alternative control, *Heterotermes sulcatus*

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The infraorder Isoptera (Krishna *et al*., 2013) includes 2,882 termite species catalogued worldwide (Constantino, 2015) in which approximately 10% are considered pests (Wood, 1996; Rouland-Lefevre, 2011). They are pests in the urban, agricultural and forest environment (Logan *et al*., 1990). Due to feeding habit (Seo *et al*., 2009), termites have become a major polyphagous agricultural pest, being found in crops such as sugar cane (*Saccharum officinarum* L.), maize (*Zea mays* L.), rice (*Oryza sativa* L.), barley (*Hordeum vulgare*) and peanuts (*Arachis hypogaea* L.) (Elango *et al*., 2012).

Subterranean termites belonging to the genera Reticulitermes, Heterotermes and Coptotermes are among the most damaging (Constantino, 2002). The genus Heterotermes have 51 species, in which 12 are considered urban and/or agricultural pests (Constantino, 2015). In South America, it is found six species, being all of them considered pests (Constantino, 2002). In Brazil, the genus Heterotermes cause damage in agricultural and urban areas. In the cultivation of sugar cane, the losses may reach 10 t ha^{-1} $year^{-1}$ (Almeida and Alves, 2009). In the Northeastern region, *H. sulcatus* is the most destructive species of subterranean termites in urban areas (Vasconcellos *et al*., 2002). Recently, *H. sulcatus* was found attacking fruit orchards in the Southern region of Piaui, Brazil, with a considerable loss in production of cashew trees.

The control of termites is made mainly through the application of synthetic insecticides or traditional wood preservatives (Lee and Ryu, 2003; Pandey *et al*., 2012). However, these insecticides have active ingredients in its composition identified with Persistent Organic Pollutants (POPs) (Verma *et al*., 2009), which although effective, when applied in a large scale contaminates the soil, can trigger resistance mechanisms of pests to pesticides and reducing the action of natural biological control agents. Thereby, it is necessary to find control strategies that are relevant to the sustainability of agricultural production, added to a philosophy based on Integrated Pest Management (IPM) (De Freitas Bueno *et al*., 2012). In this context, plant extracts stand out for constituting a rich source of bioactive molecules (Elango *et al*., 2012), with rapid degradation when compared to organochlorine isecticides, besides being obtained from renewable sources (Roel, 2001). Among the plants that present insecticides activities, the genus Croton (Euphorbiaceae) has been widely studied for constituting several active substances as terpenoids, flavonoids and alkaloids (Braga, 1960; Silva *et al*., 2012). *Croton urucurana* and its fractions semi purified showed antibacterial activity against *Bacillus subtilis* and *Escherichia coli* (Cai *et al*., 1993). The methanolic extract of *C. urucurana* bark inserted in artificial diet of larvae of *Dysdercus maurus* Distant. (Hemiptera, Pyrrhocoridae) caused 70% mortality after 13 days (Silva *et al*., 2012).

Considering the advancement of researches on the botanical insecticides and their potential for pest control and the need to find slow action products to be incorporated into baits for control of subterranean termites, the objective of this study was to evaluate the potential termiticide of the ethanolic extract of bark of *C. urucurana* and their ethanol-water, hexane and bark ethyl acetate and leaf ethyl acetate fractions against subterranean termite *H. sulcatus* (Isoptera: Rhinotermitidae).

MATERIALS AND METHODS

Termites: Subterranean termites, *H. sulcatus* were collected from corrugated cardboard traps of colonies localized in the city of Cristino Castro (PI, Brazil, 08°04'49'' S, 44°13'27'' W). Traps were brought to the laboratory and the termites were transferred to plastic boxes containing moist soil. Only large workers (third instar) were used in the experiments. Termites were used within four days after field collection.

Plant material: The leaves and bark of *C. urucurana* were collected in May 2011, in the region of Dourados, Mato Grosso do Sul, Brazil. The species was identified by Claudio Conceição, Biology Department, Federal University of Mato Grosso do Sul-UFMS, Campo Grande-MT and a sample has been deposited (no. 5009) at CGMS Herbarium.

Preparation of the extract: In the present study, 350 g of *C. urucurana* leaves and bark were collected and were separated and dried in forced air circulation oven, for three days at a temperature between 40-50°C. After drying, the materials were shredded and submitted to extraction with 5 L of ethanol (3x), by means of Buchner funnel with filter paper. The filtrate was evaporated to dryness under reduced pressure forming the crude extract. After that, the extract was submitted to chromatography in column for fractions separation. The glass column (8 cm diameter: 60 cm long) was packaged with silica gel (240 g) and a mixture of crude extract with ethanol was held to produce a reddish brown solid (424.13 g). The fractions were collected in 200 mL conical sample bottles and verified by Thin Layer Chromatography (TLC).

Anti-termitic activity: The no-choice bioassay method of Kang *et al*. (1990) and Cheng *et al*. (2007) was employed to

Table 1: Curve slopes data of concentration-mortality, LC_{50} and LC_{95}, X^2 to the crude extract *Croton urucurana* and the ethanol-water, hexane, leaf ethyl acetate and bark on *Heterotermes sulcatus*

Concentrations (ppm)	Crude extract	FEA	FHEX	FAEC	FAEFO
250	X	X			
500	X	X			
1,000	X	X	X	X	X
3,000	X	X	X	X	X
5,000	X	X	X	X	X
8,000			X	X	X
10,000				X	X

EPM: Medio standard error, LC_{50}: Lethal concentration 50%, LC_{95}: Lethal concentration 95%, CI: Confidence interval 95% probability, X^2: Chi-square, P: Probability, FEA: Fraction ethanol-water, FHEX: Hexane fraction, FAEFO: Ethyl acetate fraction leaf, FAEC: Ethyl acetate fraction bark

evaluate the anti-termitic activity of the bark extract of *C. urucurana* and their fractions. Petri dishes (9 cm in diameter and 1.5 cm high) filled with approximately 15 g of sterilized sand were used to test the toxicity of the extract against the workers of the *H. sulcatus*. Concentrations of 250, 500, 1000, 3000, 5000, 80000 and 10,0000 ppm of the extract dissolved in 900 µL of ethanol were applied to 1 g filter paper samples (Whatman No. 3 and 8.5 cm in diameter) as shown in Table 1. A blank filter paper and filter paper treated with solvent only were used as the controls. After the solvent was removed from the treated filter papers by air-drying at ambient temperature, 100 active termites (90 workers and 10 soldiers) above the third instar were added to each plate. Drops of water were periodically sprinkled onto the sterilized sand in the dishes to maintain sufficient moisture for the termites. The experimental delineation used was randomized, with six repetitions for each sample. The mortality of termites was evaluated daily for 14 days. Additionally, Lethal Concentrations-LC and Lethal Time-LT were estimated.

Data analysis: The mortality dada corrected were submitted to analysis of variance ANOVA and separated by Tukey HSD ($p<0.01$, software SISVAR 4.6). The graphics showing the percentage of survival were made using SigmaPlot software system (2.001 thousand SPSS, Chicago, IL, USA). Lethal concentrations-LC_{50} and LC_{95} and lethal time-LT_{50} were calculated by Probit through the procedure PROC PROBIT Systen of Statistical Analyses program SAS.

RESULTS

Mortality of termites: The crude extract of *C. urucurana* resulted in 100% of mortality at concentrations of 1000, 3000 and 5000 ppm in 10, 6 e 4 days, respectively. At concentrations of 250 and 500 ppm, the activity of the extract was slower but with low mortality of 36 and 35%, respectively, after 15 days (Fig. 1a). In the ethanol-water fraction, at a 3000 ppm concentration was more toxic to termites than other concentrations within five days, thereafter, at a concentration of 5000 ppm resulted in higher percentage of mortality (Fig. 1b). The hexane fraction (Fig. 1c) in 8.000 ppm concentration resulted in 50% mortality of termites after 7 days. For concentrations of 500, 1000 and 3000 ppm, the survival was above 40% (Fig. 1c). The fractions of bark ethyl acetate (Fig. 1d) and leaf ethyl acetate (Fig. 1e) presented themselves efficient in the control of termites in the concentration of 10,000 ppm. The mortality rate of 100% of termites in days 12 and 13 after application of bark ethyl acetate fraction and leaf ethyl acetate fraction, respectively (Fig. 1d and e).

Estimate of lethal concentration: The results of probit analysis for determination of lethal concentrations (LC_{50} and LC_{95}) showed that LC_{50} of ethanol-water fraction was the most efficient one. In this fraction, the minimum concentration required to cause 50% of mortality in workers was estimated in 280 ppm with an interval ranging from 235-319 ppm. As for the values of $LC_{95,}$ the ethanol-water fraction (831 ppm) also showed higher toxicity compared to other compounds (Table 2). Among the compounds tested, the leaf ethyl acetate fraction was considered less toxic, with LC_{50} estimated in 1089 ppm and LC_{95} in 4150 ppm (Table 2).

Estimating the lethal time: For the analysis of Lethal Time (LT) the crude extract of *C. urucurana* was the most toxic, with LT_{50} estimated in 2.6 days in 3,000 ppm concentration. In the same concentration ethanol-water fraction, obtained a LT_{50} estimated at 3.8 days and also showed high toxicity when compared to the control LT_{50} (93.5 days). The hexane, bark ethyl acetate, leaf ethyl acetate fractions, in 3000 ppm concentration, presented less toxicity with a LT_{50} of 14.3, 12.3 and 14 days, respectively, compared to the control (93.5 days). As for the LT_{95} the results show higher toxicity of crude extract of *C. urucurana* (5 days) compared to other compounds and lower toxicity to bark ethyl acetate (79.6 days) 3000 ppm concentration (Table 3).

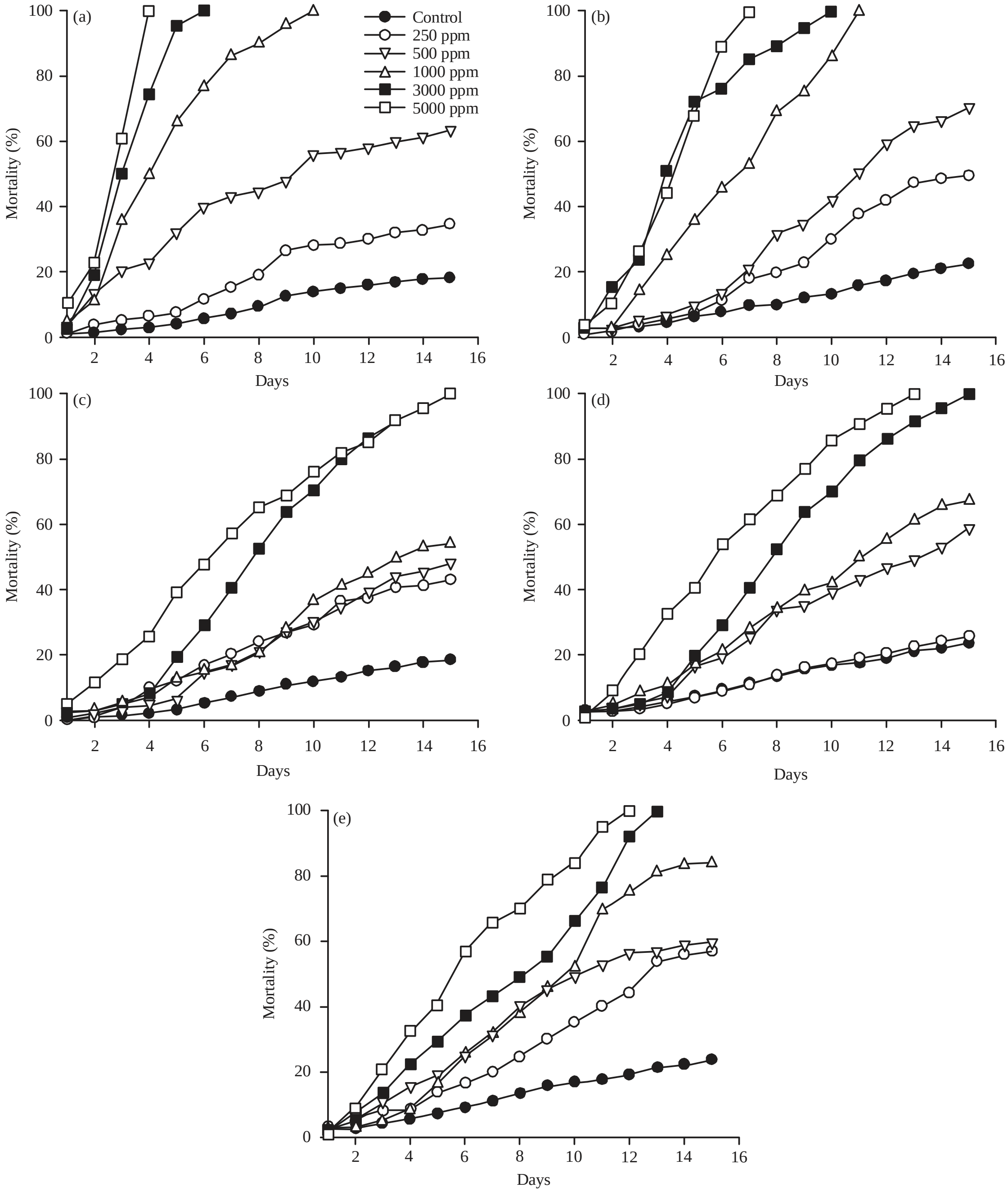

Fig. 1(a-e): Mortality of the workers of *H. sulcatus* in contact with filter paper treated with (a) Extracto crud of *C. urucurana*, (b) Fraction ethanol-water, (c) Hexane fration, (d) Leaf ethyl acetate and (e) Bark ethyl acetate

Table 2: Concentrations used in the different bioassays from the crude extract and semipurified fractions of *Croton urucurana*

Croton urucurana	Slope (EPM)	CL_{50} (95% CI)	CL_{95} (95% CI)	X^2	p-value
Crude extract	2.51±1.02	304 (244-356)	881 (724.2-1194)	71.2	0.01
FEA	1.03±1.01	280 (235-319)	831 (653.3-1300)	37.8	0.01
FHEX	1.11±1.05	589 (477-656)	933 (498.3-1392)	26.0	0.01
FAEFO	0.81±0.90	1089 (949-1805)	4150 (2982-5934)	124.7	0.01
FAEC	2.61±1.61	886 (467-1633)	2086 (1885-3535)	21.0	0.01

FEA: Ethanol-water fraction, FHEX: Hexane fraction, FAEC: Peel ethyl acetate fraction, FAEFO: Leaf ethyl acetate fraction

Table 3: Termiticide activity (LT_{50} and LT_{95}/days) of *Croton urucurana* and their fractions in the concentration of 3000 ppm on *Heterotermes sulcatus* workers

Croton urucurana	Slope (EPM)	LT_{50} (95% CI)	LT_{95} (95% CI)	X^2	p-value
Control	0.01±0.01	93.5 (66.8-148.5)	171 (82-193.8)	616.9	0.01
Crude extract	0.01±0.06	2.6 (2.5-2.7)	5.0 (4.8-5.3)	334.2	0.01
FEA	0.04±0.08	3.8 (3.5-4.1)	8.8 (8.0-9.9)	224.9	0.01
FHEX	0.02±0.02	14.3 (13.2-15.8)	68.3 (53.7-92.3)	245.4	0.01
FAEFO	0,01±0,02	12.3 (11.4-13.9)	58.0 (47.5-77.6)	276.2	0.01
FAEC	0.03±0.03	14.0 (12.5-16.3)	79.6 (56.5-128.8)	146.1	0.01

EPM: Medio standard error, LT_{50}: Lethal time 50%, LT_{99}: Lethal time 99%, IC: Confidence interval 95% probability, X^2: Chi-square, P: Probability, FEA: Fraction ethanol-water, FHEX: Hexane fraction, FAEFO: Ethyl acetate fraction leaf, FAEC: Ethyl acetate fraction bark

DISCUSSION

Survival analyses showed that the crude extract *C. urucurana* provided higher percentage of mortality of *H. sulcatus* in less time, when compared to the semipurified fractions. This fact may be related to bioactive compounds with insecticidal effect present in species of the genus Croton as the tannins. This phenolic compound binds readily with proteins to form a complex tannin-protein that reduces, significantly, the growth and survival of insects, once it turns off digestive enzymes and consequently makes it difficult the digestion (Mello and Silva-Filho, 2002). Additionally, the subterranean termites from the family Rhinotermitidae have protozoa and bacteria responsible for most of the digestion of cellulose. The reduction of these endogenous microorganisms decreases considerably the efficiency in cellulose degradation this group of termites and they can die from starvation (Lo *et al*., 2010).

Ahmed *et al.* (2006) reported that termites *Microtermes obesi* (Isoptera: Termitidae) treated with extract obtained from seeds of *Croton tiglium* L. (Euphorbiaceae) decreased number of colonies of bacteria in the intestinal flora of termites in comparison with those from untreated termites. As the intestines of Termitidae family consists of a consortium of bacteria and endogenous cellulolytic enzymes (Tokuda *et al*., 2012) responsible for the degradation of cellulose, there has been a reduction in nutrient absorption and consequently on survival.

Lethal concentration analyses showed that the ethanol-water fraction presented the lowest value of LC_{50} (280 ppm) and LC_{95} (831 ppm) and it was considered the most toxic fraction for *H. sulcatus*, followed by crude extract of *C. urucurana* with LC_{50} estimated in 304 ppm and LC_{95} of 881 ppm. These compounds showed symptoms of convulsions, tremor, muscle weakness, indicating the presence of bioactive molecules present in plant secondary metabolites, which may show neurotoxic effect. Ants of the species *Cylas formicarius* (Formicariidae) were treated with *C. cajucara*, which presented neurotoxic symptoms (Kubo *et al*., 1991). In this paper, poisoning symptoms appear rapidly in workers of *H. sulcatus*, after feeding with extract *C. urucurana*. According to these authors, the poisoning symptoms of *C. cajucara* is related to the presence of cis-desidrocrotonine, present in various species of the genus Croton and consequently, can relate to the symptoms observe in *H. sulcatus*.

According to the results in this work, the leaf ethyl acetate fraction LC_{50} (1089 ppm) was considered less toxic to *H. sulcatus* than t he bark ethyl acetate fraction LC_{50} (886 ppm). The efficiency of a botanical insecticide varies with the concentration (Silva *et al*., 2012) and the part of the plant from which the toxic metabolite was synthesized (Haas *et al*., 2002). Therefore, it is likely that the chemicals present in the bark ethyl acetate fraction have the greatest potential termiticide. Such statement is based on chemical studies carried out with the bark ethyl acetate fraction, which resulted in the isolation of phenolic compounds, catechin and gallocatechin (Winkel-Shirley, 2001), which may be involved in insecticidal activity observed on termites.

These condensed tannins are phenolic compounds which when consumed, bind the digestive proteins of insects and reduces their survival (Winkel-Shirley, 2001). In bioassays conducted with *H. sulcatus* there was total consumption of filter paper treated with these fractions, which can prove that termiticide activity observed for *H. sulcatus* is a result of the action of the compounds catechin and gallocatechin.

Silva *et al.* (2009), obtained similar results when they tested the bark ethyl acetate fraction of *C. urucurana* embedded in artificial diet of *A. kuehniella* (Lepidoptera: Pyralidae). They tested this fraction in concentration of 1.0% (10000 ppm) and reached an average of 55% mortality of *A. kuehniella*. For the larvae fed with solutions of this fraction in concentrations of 0.5% and 1.0 (5000 and 10000 ppm), there was a reduction in weight of 54 and 30%, respectively, when compared to the control treatment.

As for lethal time estimates, the crude extract of *C. urucurana* presented the lowest LT_{50} (2.6 days) and LT_{95} (5 days), in a concentration of 3,000 ppm. Ahmed *et al.* (2007), working with extract of *C. tiglium* on *Odontotermes obesus* Rambur (Isoptera: Termitidae). The presented similar effect with this work (of LT_{50}, 1.5 day). However, in higher concentrations (500,000 and 100,000 ppm), the extract of *Jatropha curcas* L. which also belongs to the family Euphorbiaceae while being tested on *O. obesus* resulted in higher LT_{50} (4 days) in a higher concentration (100,000 ppm) when compared to the crude extract of *C. urucurana* on *H. sulcatus*.

Croton urucurana and its fractions showed potential termiticide, resulting in a high mortality of *H. sulcatus*. According to Guerra (1985), the species of Croton have high power insecticide and in some cases are more toxic to insects than the pyrethrum found in chrysanthemum flowers, that offers wide spectrum of action. Thus, these compounds have high probability of being applied in controlling subterranean termites. Future studies are needed to confirm the efficiency of the compounds the *C. urucurana* against *H. sulcatus.*

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