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Original Article

LARVICIDE AND ANTIFUNGAL ACTIVITIES OF SARSAPARILLA (*SMILAX LARVATA*) EXTRACTS

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ABSTRACT

Objective: This study aimed to investigate the toxicity, antimicrobial and larvicide activities of *Smilax larvata* Griseb. (Smilacaceae) extracts.

Methods: Toxicity evaluation was performed by testing brine shrimp lethality. The presence of saponins in *S. larvata* extracts was investigated using a hemolysis test. For antimicrobial investigation, *S. larvata* extracts were submitted to disk diffusion assay, and larvicide activity was evaluated using a larvicidal assay against *Aedes aegypti* larvae.

Results: *S. larvata* extracts were not toxic to the shrimp and in the hemolysis test, only hexane, chloroform and ethanolic extracts showed significant hemolytic activity at 1000 µg. mL^{-1} (100%, 59% and 40% respectively). The crude ethanolic extract presented larvicide effect on third instar of *Aedes aegypti* larvae with LC_{50} = 225 µg. mL^{-1} and LC_{90} = 350 µg. mL^{-1}, and showed antifungal activity against *C. albicans*.

Conclusion: These results indicate that *S. larvata* extracts are a potential source of antifungal agents and new eco-friendly larvicide compounds.

Keywords: Smilacaceae, *Smilax larvata*, *Aedes aegypti*, larvicide.

INTRODUCTION

Mosquitoes are the primary source for the spread of diseases among the arthropods. Studies have been concentrated in *Aedes aegypti* species because of its role in the arboviruses transmission, responsible for yellow fever and dengue fever, diseases which are endemic in South and Central America, Asia and Africa [1]. Organophosphates and insect growth regulators are generally used for the control of mosquito larvae, however, their continuous use has demonstrated undesirable effects on non-target organisms, and their influence on the environment and human health has been the main concern [2]. Phytochemical compounds are an effective alternative. Plants produce several chemicals with biological properties, such as pesticide and insecticidal, that are relatively safe, inexpensive and readily available in many parts of the world [3].

Smilacaceae family comprises of five genera: *Anikenton, Coprosmanthus, HeteroSmilax, Nemexia* and *Smilax* [4]. The genus *Smilax* comprise of approximately 260 species[5] and is widely distributed throughout the world. Previous reports about species of this genus presented several biological activities including antibacterial, antifungal and cytotoxic[6-8]. Chemical studies using *Smilax* species revealed a range of new compounds such as steroidal saponins, furostanol saponins, sieboldogenin and other known phytochemicals such as flavonoids, polyphenols and coumarins [9-12].

Smilax larvata Griseb. (Smilacaceae) is a climbing plant endemic from Brazil [13] known in folk medicine as "unha-de-gato" and "salsaparrilha". The tea of its aerial parts is used as anti-inflammatory agent in Curitiba, Parana. In regards to *S. larvata*, it has been used in folk medicine; however, there are no studies about their biological activities. This study aims to contribute to *Smilax* genus investigation through evaluation of toxicity, antimicrobial and larvicide activity of *S. larvata* extracts.

A preliminary toxicity investigation was performed using the brine shrimp lethality test (BSLT). This bioassay is a general method of toxicity evaluation, which aims to provide a front-line screen and can be backed up by tests that are more sophisticated. The technique is rapid, simple, inexpensive and uses a small amount of test material[14]. BSLT is also used by laboratories which investigate environmental concerns for Ecotoxicology studies [15]. The larvicide potential of *S. larvata* extracts was evaluated against the third instar *Aedes aegypti* larvae. For the investigation of the antibacterial activity, a diffusion disk assay was performed against one fungi, two gram-positive and three gram-negative bacteria. Hassan and coworkers (2010)[16] reported studies that related the presence of saponins to the antibacterial effects of vegetal extracts. The *Smilax* genus is famous for containing saponins [5] and hemolysis assays can be used to detect this class of secondary metabolite [16]. Hemolysis activity was evaluated in blood agar plates and by a dilution method.

MATERIALS AND METHODS

Plant material

The aerial parts of *S. larvata* were collected in Curitiba, Paraná State, Brazil (25º26'45"S 49º20'51"W) in November 2010 and 2011. The botanical determination was performed by the biologist Osmar dos Santos Ribas in the Municipal Botanical Museum of Curitiba, where a voucher specimen was deposited (#103582).

Extracts preparation

The aerial parts were separated, dried and ground (940g) and extracted exhaustively in a Soxhlet apparatus with ethanol (6L) generating the ethanolic extract. The ethanolic extract was concentrated into 1/3 of the initial volume giving a crude ethanolic extract (EE) (145g). A portion of EE (140g) was partitioned into *n*-hexane(HE, 15g), chloroform(CLE, 3.7g), ethyl acetate (EAE, 3.4g) and hydro alcoholic (HAE, 50.5g) extracts.

Brine shrimp lethality test

This preliminary method of toxicity evaluation was performed according to Meyer and coworkers[17]. Third nauplii of *Artemia salina* were incubated with different extracts concentration (10, 100 and 1000 µg. mL^{-1}) for 24 hours. The lethal concentration (LC_{50}) was determined by counting the number of dead nauplii and data was analyzed by statistical program Probitos with 5% confidence level. This assay was performed in triplicate.

Hemolysis tests

Blood Agar plate method

Sterile paper discs were impregnated with 1000 µg of the extracts and were incubated in blood agar plates (Newprov®) at 36ºC for 24 hours. The formation of halo hemolysis indicates a positive result. Saponin R and Triton solution were used as control [18].

Measurement of hemolysis

Commercial sheep blood was manually homogenized, transferred (5 mL) to a tube and centrifuged for 5 minutes at 3000 rpm. Blood samples were washed three times with cold phosphate buffered saline (PBS: NaCl, 150 mM, KH_2PO_4 0.58 mM and Na_2PO_4 3.4 mM, pH 7.4). Supernatant was carefully removed by aspiration after each washing. The volume of washed erythrocytes that remained was suspended in the buffer solution to obtain a 10% suspension. The hemolysis test was carried out by mixing 200 µL of erythrocyte suspension with 200 µL of extracts diluted in PBS in different concentrations (100, 200, 500 and 1000 µg. mL^{-1}). The same amount of erythrocyte suspension was incubated with distilled water to be used as 100% hemolysis reference and with PBS to basal hemolysis measurement. After the incubation period, the solutions were centrifuged for 5 minutes at 3000 rpm and the hemolysis was determined by measuring the absorbance of supernatant at 540 nm. All experiments were performed in triplicate and the results were presented as a percentage of hemolysis[19].

Antimicrobial test

For the antimicrobial screening, the disk diffusion method was performed with the following commercial strains: (Gram-positive) *S. aureus* (ATCC 6538), *S. epidermidis* (ATCC 12228), (Gram-negative) *E. coli* (ATCC 25922), *P. aeruginosa (*ATCC 2785), *Agrobacterium tumefaciens* (11095, provided by Instituto Agronômico do Paraná, Londrina, Brazil), and (yeasts) *C. albicans* (ATCC 10231). Suspensions of the strains were prepared in 0.9% physiological saline and standardized according to 0.5 McFarland standard scale. All procedures were performed in aseptic conditions [20].

Disk diffusion

Microbial suspensions were seeded in duplicate on plates containing Muller-Hilton agar for bacteria(nutrient agar for *A. tumefaciens*)and Sabouraud Dextrose agar for yeasts. Stock solutions of *S. larvata* extracts were prepared at 100mg. mL^{-1} concentration and an aliquot of 10 µL were used to impregnate sterile discs (10 mm) giving a final concentration of 1mg extract/disc. The solvent was evaporated in ambient temperature and the dry discs were placed over the seeded material. The plates were incubated at 35ºC for 24 hrs in the case of bacteria (28°C for 48 hrs in case of *A. tumefaciens*) and 25ºC for 48 hrs in case of *C. albicans*. After incubation, the inhibition diameter was measured in mm. Ketoconazole (50µg) and chloramphenicol (30 µg) were used as positive controls with ethanol and methanol as negative controls [20,21].

Larvicidal assay

For the larvicide evaluation, World Health Organization protocols [22] were used with modifications. Only EE was evaluated in this test. Eggs of *A. aegypti* from Rockfeller line [23] were hatched by submerging them in chlorine-free water at 27 ± 2°C, with 80 ± 5% of relative humidity. The larvae was fed with fish food (Aldon basic, MEP 200 complex) until L_3 larval instar. Stock solutions of *S. larvata* extracts were prepared at 125, 250, 500 and 1000 µg. mL^{-1} concentrations with 0.5% dimethyl sulfoxide (DMSO) in chlorine-free water. The stock solutions were placed in containers with 15 L_3larvae. Each concentration had 45 larvae in triplicate. Aqueous solution of 0.5% DMSO was used as negative control and the insecticide Temephos (*O,O'*-(thiodi-4,1-phenylene) bis(*O,O*-dimethyl phosphorothioate) in chlorine-free water as positive control[24]. The insecticide standardization was performed according to World Health Organization protocols [22], [25], Lima and coworkers [26] and Braga and coworkers [27]. The diagnostic concentration of 0.06 mg. mL^{-1} was adopted. After 24 hrs, the number of dead larvae was counted. The larvae was considered dead when they did not respond to stimuli or when they did not rise to the surface of the solution [22]. The results were expressed as mean ± S. E. M. The statistical significance of differences between groups was performed by ANOVA, one-way, followed by Tukey test. $p<0.05$ was considered as indicative of significance. The $LC_{50}$and LC_{90}were calculated using probit analysis with a reliability interval of 95% [28].

RESULTS AND DISCUSSION

The crude ethanolic (EE), *n*-hexane (HE), chloroform (CLE), ethyl acetate (EAE) and hydroalcoholic extracts (HAE) of *S. larvata* were tested to BSLT. All extracts showed LC_{50} above 1000 µg. mL^{-1} concentration. This finding indicates a low toxicity of *S. larvata* extracts according Meyer and coworkers [17]. Previous studies in *Smilax* species toxicity were focused on *In vivo* toxicity assays, which indicated a low toxic effect of the extracts. Under the Ecotoxicology view, this result represents a low potential to harm non-target species and to content water-soluble toxins, which could contaminate the environment.

In the hemolysis tests, no extracts presented the formation of halo hemolysis in the blood agar assay, indicating a negative result. In the dilution method (fig. 1),HE showed significant hemolytic activity at 500 and 1000 µg. mL^{-1} (92.4% and 100%) followed by CLE at the same concentrations (47.8% and 58.3%). The HAE did not cause hemolysis, indicating the absence of saponins in the *S. larvata* species. This result may be useful in quality control of *S. larvata*, once the presence of saponins in other species of this genus was reported [5,9,12].

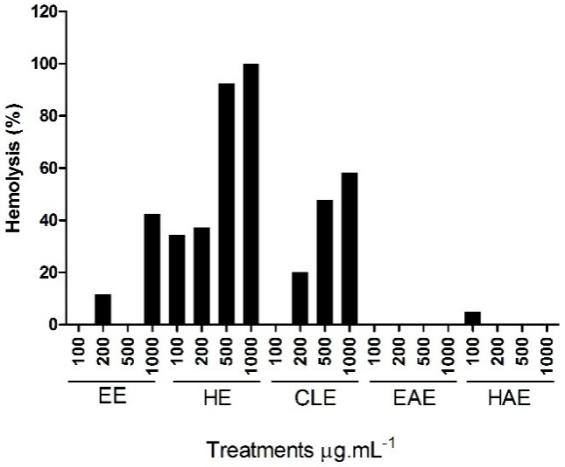

Fig. 1: Hemolytic activity in percentage of *S. larvata* extracts. EE= crude ethanolic extract; HE= *n*-hexane extract; CLE= chloroform extract; EAE= ethyl acetate extract; HAE= hidro alcoholic extract

Regarding antimicrobial activity, EE of *S. larvata* at a concentration of 1mg presented a halo inhibition (1.1 ± 0.1 cm) against *C. albicans* in disk diffusion assay. Comparing with ketoconazole 50µg (3.5 ± 0.5 cm), this extract showed a moderate anti fungal activity. Other extracts were inactive for the used strains. The antimicrobial activity of some *Smilax* species was due the presence of saponins, which presented this activity [7,31]. In *S. larvata* case, the absence of saponins indicated by hemolysis assays may explain the negative result of antimicrobial property of hydroalcoholic extract. The positive result for the EE may be due to a synergic effect of diverse phytochemical compounds, once all other extracts were inactive for the strains.

In the larvicide assay, EE of *S. larvata* presented activity against *A. aegypti* larvae with LC_{50} = 225 µg. mL^{-1} and LC_{90} = 350 µg. mL^{-1} (fig. 2, panel A). The value of LC_{50}< 500 µg. mL^{-1} indicates a toxic effect on *A. aegypti* larvae according Ciccia and coworkers (2000)[1]. It was observed a positive correlation between concentration of EE and the percentage of mortality, a trend also presented by the positive control (Figure2, panel B).

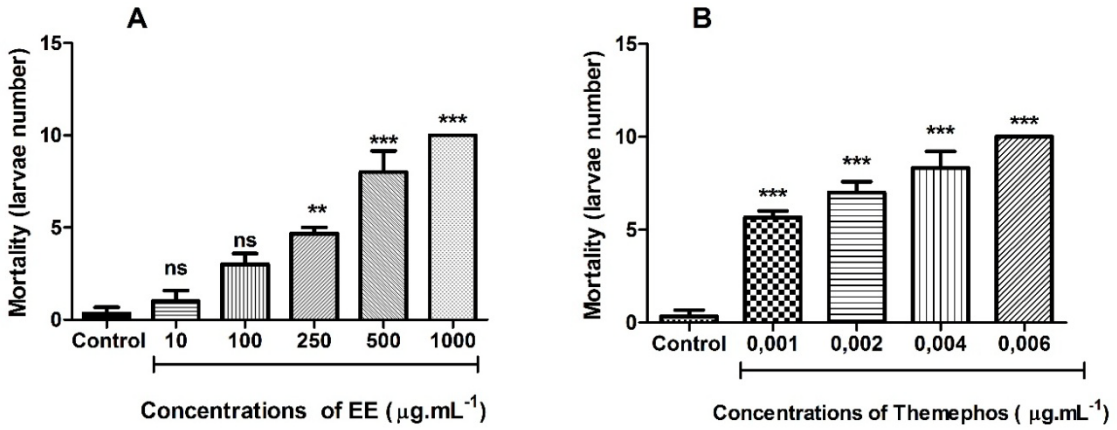

Fig. 2: Mortality in number of L3 *A. aegypti* dead larvae. A: Using the *S. larvata* crude ethanolic extract (EE). B: Using positive control themephos.*p<0.05, **<0.01, *<0.001 compared with the control group (Tukey test)**

Previous studies showed that phytochemicals are a potential source of substances that can be used in pest control programs, and several vegetal species Omit had their larvicide activity proven [3]. Larvicide activity investigation was performed against *A. aegypti* using extracts from South American plants. The authors reported a strong activity from *Abuta grandifolia* and *Minthostachys setosa* extracts [1]. Omena and coworkers (2007)also investigated this property of 51 Brazilian medicinal plants, of which two species of genus *Annona* were the most effective [24]. Regarding studies on genus *Smilax*, *S. domingensis* presented activity against *Leishmania braziliensis, L. mexicana,* and *Trypanosoma cruzi in vitro* [32]. Cytotoxic activity was reported in several species, such as *S. aspera*, *S. china* and *S. scobimicaulis* [8,9,33]. However, larvicide activity against *A. aegypti* of this genus is reported in the present study for the first time.

CONCLUSION

The use of plant extracts and its isolated substances in *A. aegypti* control is a promising alternative, which comprises decreasing noxious effects of some pesticide compounds on non-target species and on the environment. The results presented in this work suggest that the crude ethanolic extract of *S. larvata* is a potential source of an eco-friendly larvicide against *A. aegypti* larvae and antifungal agent. Further investigations on the isolation and structure determination of the substances that are responsible for the biological activities are underway.

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CONFLICT OF INTERESTS

Declared None

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