IN VITRO LARVICIDAL EVALUATION OF *Annona muricata* L., *A. diversifolia* Saff. AND *A. lutescens* Saff. EXTRACTS AGAINST *Anastrepha ludens* LARVAE (DIPTERA, TEPHRITIDAE)

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SUMMARY

Annonaceae (custard apple family) extracts have demonstrated their insecticidal potential against a variety of insect pests. This study is aimed to determine the activity of ethanolic and aqueous extracts of the stems and leaves of Annona muricata *L.,* A. diversifolia *Saff. and* A. lutescens *Saff. against larvae of* Anastrepha ludens *(Mexican fruit fly). Ethanolic extracts were obtained by Soxhlet extraction, and aqueous extracts were obtained by boiling. Larvicidal activity against third instar* A. ludens *larvae was determined after 24 and 72h of larval exposure to the extracts at concentrations of 100, 1000 and 2000µg·ml^{-1}. Additionally, toxicity was quantified by the Artemia salina (brine shrimp) assay. The results indicate that extracts of the three aforementioned* Annona *species show time-dependent larvicidal activity against* A. ludens*, with variable mortality rates at 72h of exposure, as follows:* A. lutescens *87-94%,* A. diversifolia *70-90% and* A. muricata *63-74%.*

Introduction

Annonaceae (custard apple family) plants have been intensively studied since they were discovered to contain compounds with important biological properties. These properties include cytotoxic, antitumor, antiparasitic, antifungal, antispasmodic, repellent and insecticidal activities (Cavé *et al*., 1997).

Among the reports of pest control attributed to this plant family are the assessment of the insecticidal activity of acetonic and ethanolic extracts of *Annona squamosa* L. seeds against the cowpea weevil *Callosobruchus maculates* (Dharmasena *et al*., 2001), and the demonstration of acaricidal activity of *Uvaria versicolor* and *U. klaineana* against the dust mite *Dermatophagoides pteronyssinus* (Akendengue *et al*., 2003). In addition, ingestion of and contact with methanolic extracts of *Annona muricata* seeds are lethal to first and second instar larvae of fall armyworm *Spodoptera frugiperda* (Gómez, 2005). Furthermore, *A. squamosa* extracts impart larvicidal activity, and the susceptibility of different larval instars of diamondback moth *Plutella xylostella* L. and cabbage looper *Trichoplusia ni* Hübner to these extracts have been documented by Leatemia and Isman (2004). Other studies regarding compounds isolated from Annonaceae have indicated both larvicidal and antifeedant activities (Guadaño *et al*., 2000, González-Coloma *et al*., 2002, Álvarez *et al*., 2007).

The only documented *Anastrepha* study to date was carried out by Perales and Martínez (1999). This study investigated the effect of aqueous extracts of *A. squamosa* leaves against *Anastrepha obliqua* during oviposition, hatching and adult emergence. The results indicated that the infusion of *A. squamosa* leaf extract at a concentration of 1% caused a decrease in hatching of up to 60%, whereas an extract concentration of 5% reduced adult emergence by 20%. However, the potential of extracts derived from Annonaceae species for the control of *Anastrepha ludens* (Mexican fruit fly) has not yet been demonstrated.

Annona muricata, *A. diversifolia* and *A. lutescens* are perennial tropical trees with edible fruits. These species are used almost exclusively for consumption or, in some regions, for the formation of living hedges. Coupled with this, these three species produce large amounts of cytotoxic compounds (Zafra-Polo *et al*., 1998; Abrajan, 2002; Schlie *et al*., 2009; de la Cruz *et al*., 2011), making them promising candidates in the ongoing search for natural resources with insecticid-

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EVALUACIÓN *IN VITRO* DE EXTRACTOS DE *Annona muricata* L., *A. diversifolia* saff. Y *A. lutescens* saff. SOBRE LARVAS DE *Anastrepha ludens* (DIPTERA, TEPHRITIDAE)

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RESUMEN

Los extractos de Annonaceae han demostrado tener potencial insecticida contra diferentes insectos plaga. Este trabajo tuvo como objetivo determinar la actividad de extractos etanólicos y acuosos de tallos y hojas de Annona muricata*,* A. diversifolia *y* A. lutescens *sobre larvas de* Anastrepha ludens*. Se obtuvieron extractos etanólicos por el método de Soxhlet y acuosos por ebullición. La actividad larvicida se determinó a las 24 y 72h de exposición sobre larvas de tercer estadio con concentraciones de 100, 1000 y 2000µg·ml^{-1}. Adicionalmente se cuantificó la toxicidad con el ensayo de* Artemia salina*. Los resultados señalan que los extractos de las tres especies estudiadas presentaron actividad larvicida tiempo dependiente. Las actividades (% de mortalidad) fueron significativas a las 72h de exposición con diferencias entre las especies estudiadas:* A. lutescens *87-94%, A. diversifolia 70-90% y* A. muricata *63-74%.*

AVALIAÇÃO *IN VITRO* DE EXTRATOS DE *Annona muricata* L., *A. diversifolia* saff. E *A. lutescens* saff. EM *Anastrepha ludens* (DIPTERA, TEPHRITIDAE)

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RESUMO

Os extratos de Annonaceae têm demostrado potencial inseticida contra diferentes pragas. Dentre as espécies ainda não avaliadas no controle de pragas como a Anastrepha ludens *se encontram a* Annona muricata*,* A. diversifolia *e* A. lutescens*. Este trabalho teve como objetivo determinar a atividade de extratos etanólicos e aquosos de caules e folhas destas espécies sobre larvas de* A. ludens*. Os extratos etanólicos foram obtidos pelo método de Soxhlet e o aquoso por ebulição; a atividade larvicida foi determinada durante 24 e 72h de exposição sobre larvas de terceiro estádio com concentrações de 100, 1000 e 2000µg·ml^{-1}. Adicionalmente foi quantificada a toxicidade com ensaio de* Artemia salina*. Os resultados demonstram que os extratos das três espécies estudadas apresentaram atividade larvicida dependente do tempo de exposição. Com 72h de exposição observou-se mortalidade de 87-94% com os extratos de* A. lutescens*, 70-90% com* A. diversifolia *e 63-74% com* A. muricata*.*

al/pesticidal properties. Therefore, the goal of this research was to evaluate the effectiveness of ethanolic and aqueous extracts of the stems and leaves of *A. muricata*, *A. diversifolia* and *A. lutescens* against *A. ludens* larvae.

Materials and Methods

Plant material

The plant material (stems and leaves) was collected in 2005 in Tuxtla Gutiérrez, Chiapas, Mexico. The reference specimens, *Annona diversifolia* (351), *A. lutescens* (352) and *A. muricata* (4152), were deposited at the Herbario Eizi Matuda (HEM) herbarium of the Universidad de Ciencias y Artes de Chiapas.

Extracts

The stems and leaves were dried in the shade at room temperature. The ethanolic extracts were obtained using 100g of dry powdered plant material extracted three times with absolute ethanol (400ml) in a Soxhlet extraction apparatus under reflux for 8h. Next, the ethanolic extracts were concentrated under low pressure at 40°C. The aqueous extracts were prepared by heating 200g of dry powdered plant material in 500ml of boiling distilled water for 20min. The infusion was decanted and filtered through a Buchner funnel, and the aqueous extract was concentrated until completely dry in Pyrex glass containers at room temperature. These extracts were used to prepare various concentrations of ethanolic and aqueous stem and leaf extracts, diluted in 5% ethanol or distilled water, respectively, for the evaluation of larvicidal activity.

Bioassay with Anastrepha ludens *larvae*

Evaluation of the stem and leaf extracts was conducted in the laboratories of the Subdirección de Desarrollo de Métodos of Moscafrut, SAGARPA-IICA (Secretaría de Agricultura, Ganaderia, Desarrollo Rural, Pesca, y Alimentacion - Inter-American Institute for Cooperation on Agriculture) program, located in Metapa de Domínguez, Chiapas, Mexico. Biological material was obtained from Moscafrut, located in Metapa de Domínguez, Chiapas, Mexico. The larvae were obtained from the same facility and were randomly chosen for testing.

To determine larvicidal activity, the ethanolic or aqueous extracts were incorporated into larvae feed at final concentrations of 100, 1000 and 2000µg·ml^{-1}. Fifty *A. ludens* larvae in the third instar were introduced to each concentration of stem and leaf extract. Two negative controls were used, one consisting of distilled water and the other consisting of 5% ethanol. GF-120 fruit fly bait was employed as the positive control. The data corresponding to the mortality of *A. ludens* larvae were recorded after 24 and 72h of exposure to the stem and leaf extracts. Five replicates of the experiment were performed, and the results were adjusted according to Abbott´s formula (Bobadilla *et al*., 2002).

All experimental data for larvicidal activity were expressed as means ± SD (standard deviation) for the five experimental replicates. To ascertain the differences in larvicidal activity between the various extracts, an analysis of variance (ANOVA) was performed. Significant differences between means

were determined by Bonferroni's multiple comparison test. P values <0.05 were regarded as significant. Finally, a paired-sample analysis was performed to establish whether the larvicidal activity was more closely related to the type of extract (ethanolic *vs* aqueous) or to a particular plant organ (stem *vs* leaf).

Anastrepha ludens *larvae diet*

In all assays, the larvae diet consisted of a mixture of 18.0% corncob powder, 9.1% sugar, 7.0% yeast, 5.3% cornmeal, 0.2% nipagin sodium, 0.4% sodium benzoate, 0.4% citric acid and 0.1% Guar gum.

Artemia salina *bioassay*

The toxicity level of all extracts was evaluated by using the brine shrimp (*Artemia salina*) bioassay, as described by Meyer *et al.* (1982) and McLaughlin *et al.* (1998). To obtain *A. salina* larvae, eggs (20mg) were incubated in saline solution (250ml; 25% artificial sea salt CORALIFE® dissolved in distilled water) for 48h under artificial light and at room temperature in a small pond divided into two compartments, one dark and one illuminated. Eggs were deposited in the dark side of the pond. At hatching, the larvae migrated to the illuminated side of the pond and from there were collected for testing.

Four concentrations (1, 10, 100 and 1000µg·ml^{-1}) of each extract were prepared in 5ml of artificial saline medium, and 10 crustacean larvae were transferred into vials containing each of the plant samples to be evaluated. The negative control was a sample of artificial saline medium containing no plant extract. Incubation of the larvae with the extracts or the negative control was conducted at room temperature. The number of dead larvae was counted at 6 and 24h, adjusting for dead larvae in the negative control according to Abbott's formula (Bobadilla *et al.,* 2002). Five experimental replications were performed for each concentration of each plant extract. LC_{50} (half-maximal lethal concentration) values for each extract were determined using a Probit regression analysis to determine the 95% confidence interval. LC_{50} values >1000µg·ml^{-1} for all plant extracts were considered inactive. All statistical analyses for toxicity were performed using Stata 8.0 statistical software.

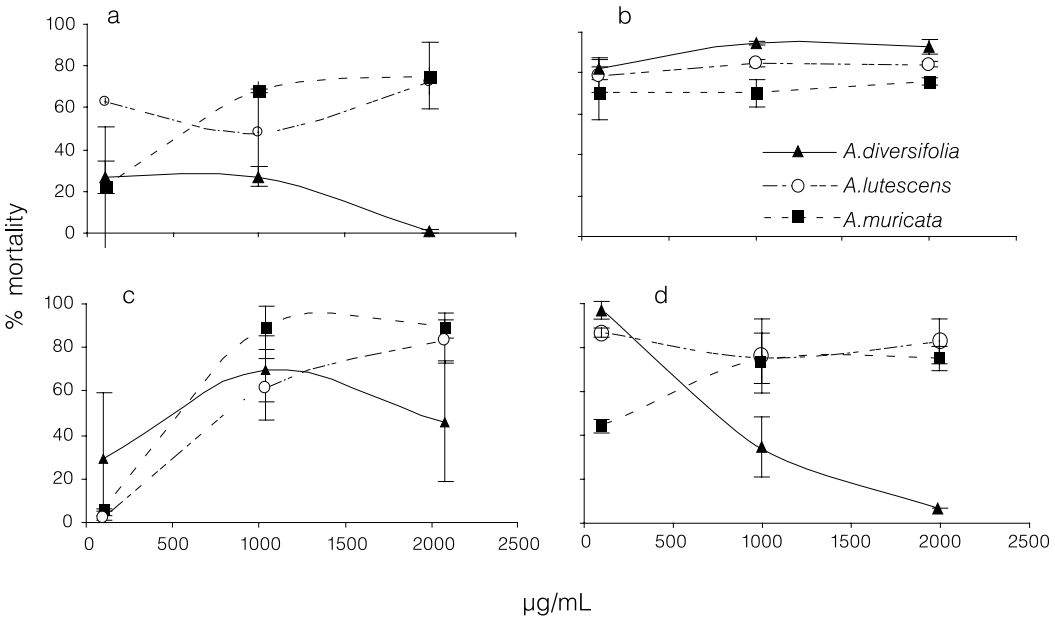

Figure 1. Larvicidal activity of *Annona* spp. extracts against third instar larvae of *A. ludens*. a: ethanolic leaf, b: aqueous leaf, c: ethanolic stem and d: aqueous stem extracts. The data were collected after 72h of larval exposure to the extracts. Mortality percentages for *A. ludens* larvae are given as means ±SD.

Results

To study the larvicidal potential of ethanolic and aqueous extracts of the stems and leaves of *Annona muricata*, *A. diversifolia* and *A. lutescens*, all extracts were evaluated against *Anastrepha ludens* larvae at 72h. Among the ethanolic leaf extracts, the most active extracts, in terms of the percentage of larvae killed at the concentration of 2000µg·ml^{-1}, were those derived from *A. diversifolia* (75.3%) and *A. muricata* (72.8%); P<0.01), while the greatest mortality rates stemming from the *A. lutescens* extracts occurred at 100µg·ml^{-1} (Figure 1a). The aqueous leaf extracts of all three species were more effective than the corresponding ethanolic extracts and showed similar activity at 72h: *A. lutescens* 92.8%; *A. muricata* 84.3%, and *A. diversifolia* 75.8% at 2000µg·ml^{-1}; P<0.01 (Figure 1b).

The results with the ethanolic stem extracts indicated that the activities of the *A. diversifolia* and *A. muricata* extracts were somewhat concentration-dependent (Figure 1c), in that the 2000µg·ml^{-1} concentration was more efficacious (90.0% and 82.8% larvicidal activity at 2000µg·ml^{-1}, respectively; P<0.01) than either the 100 or the 1000µg·ml^{-1} concentration. The same concentration of ethanolic *A. lutescens* stem extract showed lower larvicidal activity (30.9%) compared with the other ethanolic stem extracts. With respect to the aqueous stem extracts, *A. lutescens* and *A. muricata* extracts at the 100µg·ml^{-1} concentration had the highest larvicidal activity (95.9% and 86.0%, respectively; P<0.01). However, the activity of the aqueous *A. lutescens* extract decreased with increasing extract concentration (Figure 1d).

Two extract concentrations, 100 and 1000µg·ml^{-1}, were selected to determine the effect of exposure time to all plant extracts (ethanolic versus aqueous, stem versus leaf) on larvicidal activity. The activity of all plant extracts at 24h was relatively minor, yielding only ~5.4% mortality. However, the larvicidal activity increased significantly at 72h (Table I). To determine whether the larvicidal activity was more closely related to the type of plant extract (ethanolic *vs* aqueous) or the organ (stem *vs* leaf), appropriate statistical analyses were performed (Table II). Significant differences were observed in both cases for *A. lutescens*. For *A. diversifolia*, a significant difference was found between the stem and leaf extract for the ethanolic but not the aqueous extract. By contrast, no statistically significant differences were found between any of the extract activities for *A. muricata*, although, as

noted above, the aqueous leaf extract tended to be more effective than the ethanolic leaf extract only at 100µg·ml^{-1} (Table I).

The results from the *A. salina* bioassay revealed that for all three *Annona* species, the toxicity of each ethanolic extract was higher than that of the corresponding aqueous extract. For example, the LC_{50} values for the ethanolic *A. diversifolia* leaf and stem extracts were 52.0 and 409.1µg·ml^{-1}, respectively, after 24h of larval exposure. Aqueous extracts, with the exception of the stem extract derived from *A. lutescens*, were comparatively less toxic (Table III).

TABLE I
LARVICIDAL ACTIVITY OF *Annona* SPP. EXTRACTS AGAINST THIRD INSTAR LARVAE OF *A. ludens* AT DIFFERENT EXPOSURE TIMES (24 *vs* 72h)

Specie	Extract	Tissue	Mortality (%) 100µg·ml^{-1}		Mortality (%) 1000µg·ml^{-1}	
			Exposures time			
			24h	72h	24h	72h
A. diversifolia	Ethanolic	Leaves	1.0 ±1.0	22.0 ±30	2.2 ±1.8	68.3 ±1.4*
	Ethanolic	Stems	1.2 ±1.3	5.8 ±0.7	1.5 ±2.4	89.3 ±14.1*
	Aqueous	Leaves	0.0 ±0	70.3 ±18.4*	2.4 ±2.6	70.3 ±8.0*
	Aqueous	Stems	0.2 ±0.4	44.3 ±4.2*	0.8 ±0.8	74.3 ±14.0*
A. lutescens	Ethanolic	Leaves	2.4 ±2.1	27.0 ±7.7	1.6 ±1.9	27.0 ±4.9
	Ethanolic	Stems	0.2 ±0.4	29.5 ±1.5*	0.4 ±0.9	70.3 ±14.9*
	Aqueous	Leaves	1.0 ±1.7	81.7 ±4.9*	2.2 ±1.8	94.4 ±1*
	Aqueous	Stems	5.4 ±4.4	95.9 ±3.8*	1.8 ±2.9	34.1 ±13.4*
A. muricata	Ethanolic	Leaves	1.4 ±0.9	63.3 ±3.5*	0.2 ±0.4	48.3 ±63*
	Ethanolic	Stems	1.0 ±1.2	2.0 ±1.5	0.2 ±0.5	61.3 ±19.8*
	Aqueous	Leaves	0.6 ±0.9	78.3 ±12*	1.4 ±2.1	84.8 ±2.1*
	Aqueous	Stems	1.0 ±0.71	86.0 ±2.8*	1.0 ±1	75.5±23.3*

Mortality percentages are given as means ±SD. The values followed by an asterisk are significantly different (P<0.05) by an F test (Bonferroni 95% confidence interval).

TABLE II
COMPARISON OF EXTRACTS BY STATISTICAL ANALYSIS

	Aqueous leaf & stem	Ethanolic leaf & stem	Leaf aqueous & ethanolic	Stem aqueous & ethanolic
A. diversifolia	t= -0.6122	t= -3.4163	t= 0.4427	t= -1.68631
	P= 0.5574	P= 0.0091	P= 0.6697	P= 0.1302
A. lutescens	t= 10.0752	t= -6.3222	t=31.0563	t= -4.0928
	P= 0.0000	P= 0.0002	P= 1.2 E-9	P= 0.0035
A. muricata	t= 0.9512	t= -0.5515	t= 1.6381	t= 1.08089
	P= 0.3694	P= 0.5964	P= 0.1400	P= 0.3113

P<0.05 indicates significant differences.

TABLE III
REGRESSION PARAMETERS OF PROBIT ANALYSIS FOR THE MORTALITY (EXPRESSED IN TERMS OF LC_{50} AND LC_{90} (µg·ml^{-1})) OF *A. salina* LARVAE FOLLOWING EXPOSURE TO *Annona* SPECIES EXTRACTS

			CL_{50}	Inf. limit	Sup. limit	CL_{90}	Inf. limit	Sup. limit
A. diversifolia	Aqueous	Stem	588.685	113.46	914.262	2898.4	2184.89	4806.1
	Aqueous	Leaf	>1000			11407		
	Ethanolic	Stem	409.139	206.39	568.503	1412.97	1174.94	1825.79
	Ethanolic	Leaf	52.0284	-1089.4	478.278	2726.5	1971.26	5268.83
A. lutescens	Aqueous	Stem	1016.61	893.93	1160.13	341.84	138.682	488.207
	Aqueous	Leaf	>1000			707.00		
	Ethanolic	Stem	7115.48	1479.1		1<		
	Ethanolic	Leaf	318.972	-26.239	546.138	1<		
A. muricata	Aqueous	Stem	>1000			3984.2		2340.18
	Aqueous	Leaf	>1000			3852.6	1565.16	
	Ethanolic	Stem	865	113.92	1564.45	4539	2966.69	13851.1
	Ethanolic	Leaf	831.445	642.44	1024.48	2058.3	1735.15	2606.81

LC_{50}: lethal concentration to cause 50% mortality in population, LC: lethal concentration to cause 90% mortality in population, CI: confidence interval.

Discussion

Ecosystem deterioration is caused by the inappropriate use of insecticides and pesticides and has stimulated the search for new biodegradable alternatives for pest management. This study therefore evaluated the activity of three *Annona* species against third instar *Anastrepha ludens* larvae. Ethanolic and aqueous extracts derived from *Annona muricata*, *A. diversifolia* and *A. lutescens* showed time-dependent larvicidal potential, with significantly higher mortality of larvae at 72 *vs* 24h. The aqueous extracts from *A. lutescens* stand out in this regard, with activities reaching 95.9% (stem extract at 100µg·ml^{-1}) and 94.4% (leaf extract at 1000µg·ml^{-1}). By comparison, the larvicidal activities of the aqueous extracts derived from *A. muricata* were 86% (stem extract at 100µg·ml^{-1}) and 84.8% (leaf extract at 1000µg·ml^{-1}), whereas the larvicidal activity of the ethanolic stem extract derived from *A. diversifolia* was 89% at 1000µg·ml^{-1}.

Similar larvicidal activity levels were previously found with aqueous leaf and stem extracts of *A. muricata* against fourth instar *Aedes aegypti* larvae after 40h of exposure, but at 10-fold higher concentrations (Bobadilla *et al*., 2005). Likewise, the larvicidal activity described in this study is superior to that demonstrated with ethanolic extracts of *Annona triloba* branches against the Mexican bean beetle *Epilachna varivestis* (60% mortality, 100ppm) after 72h of exposure (Johnson *et al*.,

1996). However, with the exception of *A. lutescens* and *A. diversifolia* (limited to ethanolic stem *vs* leaf extract for the latter), the analysis of variance (95% confidence) employed in the current study did not distinguish between the larvicidal activity of aqueous stem, aqueous leaf, ethanolic stem or ethanolic leaf extracts.

Possession of similar larvicidal activity by most of the extracts employed herein could be related to both the common polarity of the ethanol and water solvents (both polar), as well as to the broad distribution of cytotoxic compounds in numerous plant organs. For example, various alkaloids and acetogenins have been reported in the stems and leaves of *Annona* species (*A. muricata* in particular), as well as in their roots and seeds (Leboeuf *et al*., 1982; Cavé *et al*., 1997; Fang-Rong *et al*., 2000). Larvicidal potential has been attributed to acetogenins due to their potent inhibitory activity against mitochondrial respiratory complex I. This inhibition was first described in the mitochondria of the intestinal cells of fifth instar European corn borer *Ostrinia nubilalis* larvae using asimicine (a polyketide; Lewis *et al*., 1993), and in the mitochondria of tobacco hookworm *Manduca sexta* larvae using bullatacine (an antitumor acetogenin; Ahammadsahib *et al*., 1993). Furthermore, benzylisoquinoline alkaloids derived from *Annona* species are characterized by their inhibitory activity against topoisomerase II (Woo *et al*., 1997), which is another potential larvicidal mechanism of the plant extracts described in this study.

The extracts derived from *A. lutescens* showed a marked difference in larvicidal activity relative to the extracts derived from *A. muricata* and *A. diversifolia*. Notably, lower *A. lutescens* extract doses were more active against *A. ludens* larvae compared with higher doses. This is suggestive of an antifeedant effect, already noted in the activity of *Asimina triloba* ethanolic extracts and an annonaceous acetogenin against *O. nubilalis* fourth instar larvae (Lewis *et al*., 1993). Liriodenine alkaloid and cherimoline-2 and rolliniastatine-2 acetogenins have all been isolated from *A. lutescens* (Abraján, 2002), which may account for the antifeedant and larvicidal activities observed for this plant species. In the present study, *A. lutescens* extracts were also highly toxic against *A. salina*. If a possible antifeedant or a deterrent-type activity is considered, it might be hypothesized that an aliment with a low concentration extract rather than one with a high concentration would be preferable so as to avoid potential cytotoxicity.

As noted above, the larvicidal/insecticidal activity of Annonaceae extracts might be related to their content of acetogenins, as evidenced by reports of the antifeedant, larvicidal, pupicidal and pesticidal effects of these compounds. Moreover, the toxicity of certain acetogenins can be selective for a particular insect; for example, annonacine exhibits antifeedant activity against the Colorado potato beetle *Leptinotarsa decemlineata*, while squamocine exhibits insecticidal activity against *L. decemlineata* and green peach aphid *Myzus persicae* (Guadaño *et al*., 2000). On the other hand, an acetogenin can have various targets; for example, in addition to their above-noted targets, squamocine and asimicine are also toxic to the melon aphid *Aphis gossypii* and the bean beetle *Epilachna varivestis* (Cavé *et al*., 1997). In either case, it can be inferred that larvicidal properties of *A. muricata*, *A. diversifolia* and *A. lutescens* against *A. ludens* are due, in large part, to their content of acetogenins, but the contribution of *Annona* species-derived bioactive alkaloids cannot be overlooked.

When analyzing the toxicity of the plant extracts against *A. salina*, a close relationship was found between toxicity and extract concentration after 24h of exposure for all three plant species. Ethanolic extracts of the leaves and stems were the most toxic, highlighting ethanolic *A. diversifolia* leaf and stem extracts (LC_{50}= 52.0 and 409.1μg·ml^{-1}, respectively) and ethanolic *A. lutescens* leaf extract (LC_{50} = 319.0μg·ml^{-1}). However, the extracts were not toxic to *A. ludens* larvae at this exposure time, which indicates a type of species-specific activity and throws into question the value of this assay as a toxicity indicator for *A. ludens*.

Conclusions

Extracts derived from the *Annona* species *A. muricata*, *A. diversifolia* and *A. lutescens* all showed time-dependent larvicidal activity against third instar *A. ludens* larvae. However, with the exception of *A. lutescens* extracts, the results of this study showed that neither aqueous *vs* ethanolic extracts, nor stem *vs* leaf extracts will figure predominantly in the development of environmentally friendly, *Annona* based insecticidal strategies to control *A. ludens*. The effects of these extracts must also be demonstrated in *A. ludens* adults and pupae, which represent the exposed stages of this pest. In conclusion, the three *Annona* species studied in this work constitute an extremely valuable, commercially feasible natural resource, due to their larvicidal activity and high content of readily attainable acetogenins and alkaloids.

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