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IN VITRO EVALUATION OF SOTOL VINASSE AS A POTENTIAL BIORATIONAL INSECTICIDE USING *Galleria mellonella* (L.) AS A MODEL INSECT

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ABSTRACT

New technologies in control pest arise as a necessity to reduce the use of agrochemical pesticides and their potential environmental risks. Therefore, it is necessary to apply standardized techniques to validate the efficiency of these products. A bioassay was conducted to determine the median lethal dose (LD_{50}) and median lethal time (LT_{50}) of sotol vinasse using *Galleria mellonella* (wax moth), as a model insect. Mortality was measured at concentrations of 50%, 75%, and 100%, and between time intervals of 0, 8, 16, 24, 48, 96, 104, 112, 120, and 128 hours. In addition, an assay was performed by adding 0.01% Tween 80® surfactant to the vinasse to observe if there were significant differences between the two treatments. The results show that the treatment with the highest mortality was 100% vinasse with Tween 80®, with a mortality of 83.33% at 48 hours post-application. The LD_{50} for raw vinasse was 72.94±6.66%, and for vinasse + Tween 80®, it was 43.88±8.39%. The median lethal time for T100+Tween 80® was 22.14 hours, for T75+Tween 80® it was 35.84 hours, for T50+Tween 80® it was 36.92 hours, T100 was 124.12 hours, T75 was 125.14 hours, and T50 was 139 hours. Significant differences were found between the treatments of raw vinasse and vinasse+Tween 80®; the addition of the surfactant positively enhanced vinasse efficiency. It is concluded that sotol vinasse performs very well against the model insect, and the bioassay allows establishing the concentration gradients and times at which it is necessary to apply this product.

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Keywords: Bioinsecticide, sotol, LD_{50}, LT_{50}, wax moth, vinasse

Introduction

The growing awareness of the environmental risks associated with the indiscriminate use of agrochemicals has led to a shift in the perception of pest control, driven by an increase in environmental consciousness [1]. This need drives the search for new technologies, in this context, the use of alternative insecticides aims to guarantee efficiency in pest control while safeguarding biodiversity and ecosystem health. Since this is a new but rapidly growing field of study, it is imperative to conduct studies that validate these new technologies, in order to know and establish the limits within which it can be considered an efficient bioinsecticide product [2].

In vitro assays or bioassays are conducted with living organisms in a controlled environment. These organisms may include cell cultures, bacteria, fungi, insects, plants, and even small animals, referred to as model organisms. Among the results assessed in bioassays is the measurement of toxicity or mortality. Moreover, these assays serve as a valuable tool in agriculture as they provide an opportunity to evaluate pest controllers, their effects, and concentrations concerning pathogenic organisms. These assays serve as a preliminary step to determine the relevance of investigating whether a particular substance can be used in animals, humans, or plants [2].

Among model organisms, the pest *Galleria mellonella* has gained relevance due to its ease to reproduce species with a short life cycle [3]. *G. mellonella*, an insect of the Lepidoptera order, is widely used in its larval state as a successful experimental model for investigating host-pathogen interactions [4].

In this research, sotol vinasse was selected to assess its performance as an insecticide. Vinasse is a by-product of alcohol production through fermentation using the sotol plant (*Dasylirium* spp) as raw material [5]. This residue is the liquid resulting after separating fermented molasses and alcohol in the distillation stage. A study published by Aviña-Ruelas et al., 2023 [6], revealed that sotol vinasse is characterized by an acidic pH (3.6-4.15), a high total solids content of 40,251.67 mgL^{-1}, melanoidins ranging from 15 to 24 mgL-1, and phenols between 700-900 mgL^{-1}. In addition, it contains micronutrients such as Ca (1052.7 mgL^{-1}), Mg (322.8 mgL^{-1}), K (7378 mgL^{-1}), and P (495 mgL-1), making it a recalcitrant contaminant when disposed of in water and soil without any treatment. However, these "negative" properties of vinasses have been investigated as potential insecticides in agricultural and forestry pests [7]. Phenols, for instance, are active compounds present in pesticides capable of inhibiting vital functions such as respiration in insects, fungi, and bacteria [8]. Furthermore, micronutrients such as calcium, magnesium, phosphorus, and potassium contribute to strengthening plants against pest attacks, either with a repellent, insecticidal, or antifungal action [9].

Successful applications of vinasses include, for example, control of earthworms in radish crops [10], control of *Oregmopyga peruviana* (red bug) in vineyards [11], and inhibition of phytopathogenic fungi like *Fusarium oxysporum* [12]. A study conducted in 2015 determined a vinasse dose of $90m^3$ per hectare that achieved good results in termite control (*Nasutitermes* spp) [13]. This research revealed a potential cost savings of up to 50% in the application of traditional insecticides.

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Surfactants are commonly used in synergy with biorational insecticides to add stability to emulsions. Tween 80® surfactant is a polyoxyethylene-80-sorbitan monooleate with hydrophilic and hydrophobic properties, which improves the miscibility of components and aids dispersion in plants [14]. Moreover, it reduces the surface tension of liquids, making it highly efficient at low concentrations, particularly when applied to enter the bodies of pests [15].

The objective of this research is to compare the effect of different sotol vinasse formulations using *Galleria mellonella* larvae as a model organism. To determine their efficiency, the Median Lethal Dose (LD_{50}), or the dose that produces 50% mortality in the population, and the Median Lethal Time (LT_{50}), which is the exposure time required for a substance to produce 50% mortality in a given population [16], were evaluated. These parameters serve as criteria for assessing the effectiveness of an insecticide [17].

Materials and methods

Selection of biorational insecticide

Sotol vinasse was selected from a local producer from the municipality of Nombre de Dios, Durango, México. Approximately 40 Liters of vinasse were extracted directly from the waste vat, the sample corresponds to one day of production, which were deposited in 20 L plastic drums. The determination of physicochemical and organic characteristics of the liquid was carried out in the laboratories of the Institute of Forestry and Wood Industry (ISIMA) of the Juárez University of the State of Durango (UJED) and Polytechnic University of Durango [6].

Model insect selection

As model insect, 600 eggs of *Galleria mellonella* (L.), acquired from "César Food's" company located in the city of Saltillo, Coahuila, Mexico, were used. Once the eggs hatched, the colony was established, and the larvae were fed with a formulated diet consisting of 25% corn flour, 12.5% wheat flour, 12.5% wheat bran, 6.25% instant yeast, 6.25% powdered milk, and 37.5% honey [18]. They were maintained at a temperature between 25 to 30°C in plastic boxes with ventilation under semi-shaded conditions, and feeding was performed every 5 days.

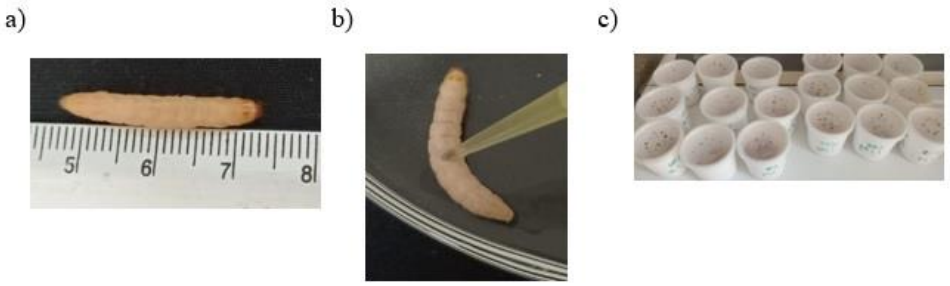

Fig. 1. Images of Galleria mellonella larvae used in this essay
a. Larvae of *G. mellonella* used in the trail.
b. Application of the treatment to the larvae.
c. Experimental units.

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At the beginning of the experiment, 300 larvae of 18 cm were selected, any larvae displaying abnormalities, such as color or texture alterations caused by parasities, were excluded from the study. Low-density beakers with pierced lids were used as the experimental unit (Fig. a,b,c).

Susceptibility of larvae to sotol vinasse (LD_{50} and LT_{50})

Three doses were used, which are the minimum suggested in bioassays to determine LD_{50} and LT_{50} [13]. Since the insecticidal action of vinasse is by contact, the assay was divided into two parts. Table 1 shows the treatment and its code.

Table 1 Treatments for in vitro tests

Treatment code	**Description** **Concentration of vinasse**
T100	100% vinasse
T75	75% vinasse
T50	50% vinasse
T100+Tween 80®	100 % vinasse +tween 80® 0.01%
T75+Tween 80®	75% vinasse + tween 80® 0.01%
T50+Tween 80®	50% vinasse + tween 80® 0.01%
Control	Distilled water
Control +Tween 80®	Distilled water+ tween 80® 0.01%

The vinasse was used without any previous treatment, and dilutions were performed with distilled water only. Treatments with emulsifier include Tween 80® at a concentration of 0.01%. The implemented doses were 50%, 75% and 100%. Three replicates consisting of three experimental units with 10 individuals in each unit and two experimental unit controls: one with distilled water only and the other with Tween 80® at 0.01% diluted with distilled water.

Applications were made topically on the dorsum of larvae, with a volume of 0.1 mL per larvae. Individuals were placed in polyethylene cups with the prepared diet.

Fig. 2. Change in larval coloration as a criterion to determine mortality

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The criterion for determining the percentage of mortality was immobility or lack of reaction when pressed with a brush [19], in addition to the change from brown to black coloration (Fig.2) [20].

Statistical analysis

A completely randomized experimental design was used.The Shapiro-Willk test was used to analyze whetehr the data on mortality percentage and lethal time followed a normal distribution. Then, an ANOVA was used to determine if there were statistically significant differences between treatmentes and the control. Finally, a Tukey test was performed to estimate which treatmentes were different from each other [21].

Method for estimating LD_{50}

For the estimations, the open-access R software (RStudio®, 2023) for Windows® was employed using the drc package version 3.0-1 and the drm function, which is a model for the analysis of concentration/dose/time/effect data [22]. This function relies on a general optimizer to minimize the negative log-likelihood function. For a continuous response, this simplifies to the least squares estimation. Model fitness threshold was assessed using the *neil.test* function. This function can be applied in regression models for both replicated and non-replicated concentrations. The model fit is considered good when it is not significant [23].

Estimation of LT_{50}

PROBIT analysis was utilized to determine the median lethal time at different doses. This type of test is efficient in data management, especially in insecticide tests and biological assays. Percentage data are transformed into probit units, which are then queried in a table of units with the same name [24].

Results and discussion

LD_{50} determination

Table 2 shows the average mortality data by dose, the treatment with the highest mortality is the vinasse at a concentration of 100%+Tween80® with a 83.3% mortality; most of the treatments did not show significant differences between their means; however, the lowest mortalities occurred in raw vinasse treatments with the 50% and 75% concentration.

Table 2. Accumulative mortality [%] of vinasse vs *Galleria mellonella* in each of the treatmentes and their corresponding statistical indicators

		Vinasse concentration [%]			
Treatment	N	50	75	100	Control
Raw vinasse	90	36.66 b	51.11b	57.77 ab¥	0 c
Vinasse+Tween 80®	90	61.11 ab	61.11 ab	83.33 a	0.22 c

n: Number of individuals.

¥: means with common letter fot the same row are not significantly different (P<0.05)

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Figure 3 shows as the most effective treatment with a higher median compared to the other treatments; although, treatments T100, T50+ Tween 80®and T75+ Tween 80® have approximately the same median, therefore, these treatments are not significantly different ($p<0.05$, Tukey) with respect to the concentration of vinasse.

Moreover, the control treatments in both cases are close to or equal to 0, which explains that the surfactant Tween 80® at a concentration of 0.01% does not act as an insecticide by itself.

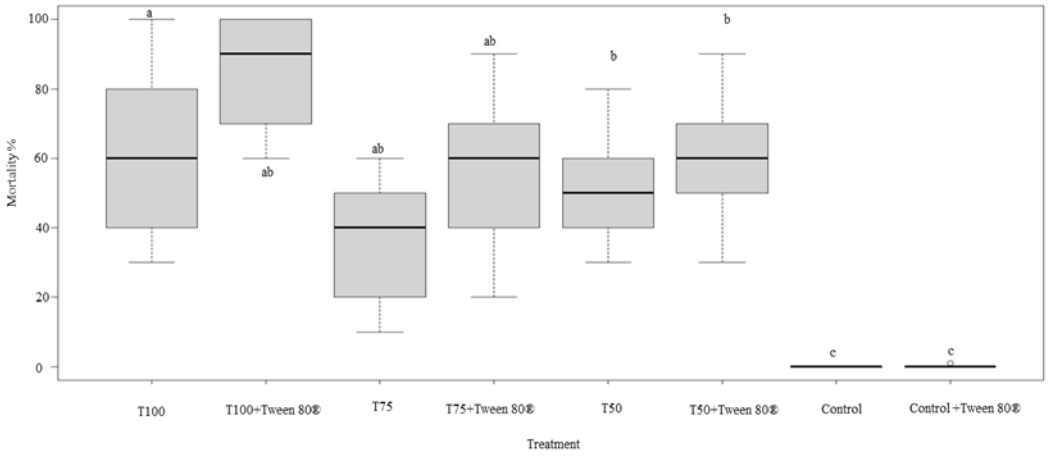

Figure 3. Comparison of mortality [%] of Galleria mellonella with respect of sotol vinasse. Different letters indicate significant difference ($p<0.05$ Tukey

Table 3 presents the median lethal dose (LD_{50}) according to each treatment. The most effective dose is vinasse+Tween 80®. It is established that a concentration of vinasse of 43.88% plus surfactant is enough to kill 50% of larvae. On the other hand, raw vinasse needs a 72.94% of concentration in order to eliminate 50% of larvae.

Table 3. Median lethal dose of sotol vinasse against *Galleria mellonella* larvae

Treatment	LD_{50} [%]	Confident [%]	Neill test p^*
T100+ Tween 80®	43.88±8.39	95	0.23
T100	72.94±6.66	95	0.99

* Neill test is significant if $p>0.05$

These results show greater efficiency in relation to biological insecticides based on entomopathogenic fungi, as in the case of application of *Cordyceps javanica* on *Galleria mellonella*, which reported a mortality rate of no more than 30% at a LD_{50} of $1.23x10^8$ conidia.mL^{-1}[18]. In contrast, the use of surfactants combined with pesticides such as Levo®, based on alkaloids like oxamatrine, decreased its efficiency by 38% for the control of wax moth larvae [25], a point to consider when deciding to mix this type of insecticides with surfactants.

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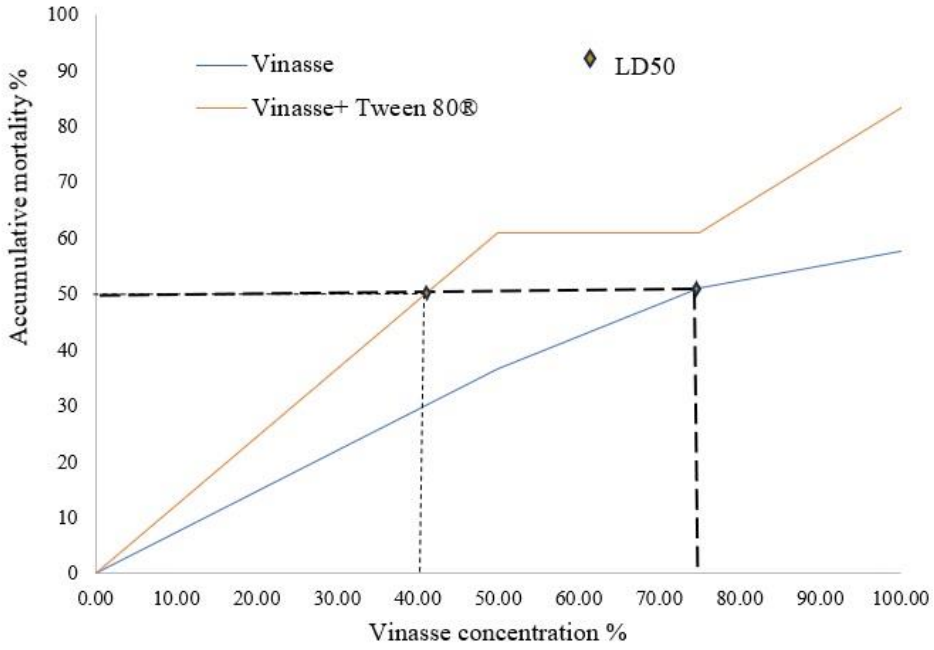

Fig. 4. Graphic representation of LD_{50} respect to raw vinasse and vinasse+Tween 80®

Figure 4 illustrates the determination of the LD_{50}. The addition of Tween 80® surfactant allows for the use of approximately 30% less vinasse to achieve efficient control of the larva.

Table 4 shows all different LT_{50} estimations where the most efficient treatment is T100+Tween80® with a LT_{50} of 22.14 hours. Similar to mortality, it is observed that the median lethal times of raw sotol vinasse are approximately 6 times slower than those applied with 0.01% Tween 80.

Table 4. LT_{50} of sotol vinasse applied on *Galleria mellonella* larva

Treatment	LT_{50} [hours]	Equation	R^2
T100+ Tween 80®	22.14	y = 1.2497x + 1.1292	0.9996
T75+ Tween 80®	35.84	y = 1.3694x + 0.099	0.9463
T50+ Tween 80®	36.92	y = 1.7864x - 0.3042	0.9495
T100	124.12	y = 5.4685x - 21.365	0.9653
T75	125.14	y = 15.476x - 69.74	0.8446
T50	139.10	y = 5.3803x - 21.553	0.9650

LT_{50}: Median Lethal Time
R^2: Adjusted R-square

For the calculation of mortality with respect to time, it was obtained that 61.11% of the individuals are dead at a time of 128 hours using T100 treatment. A mortaltity of 83.33% with a T100+ Tween 80® at a time of 48 hours. Table 5 shows the results of the behavior of sotol vinasse at different concentrations and times.

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Table 5. Mortality [%] of Galleria mellonella using different concentrations of sotol vinasse

	Treatment					
time[hours]	**T100+ Tween 80®**	**T100**	**T75+ Tween 80®**	**T75**	**T50+ Tween 80®**	**T50**
	Average mortality [%]					
0	0c	0	0c	0	0c	0c
8	10b	0	1.11b	0	8.89b	0
16	22.2ab	0	12.22ab	0	25.56ab	0
24	55.56a	0	32.22ab	0	63.33a	0
48	83.33a	0	61.11a	0	57.77a	0
96	-	10	-	0	-	2.22f
104	-	15.55f	-	4.44f	-	8.89f
112	-	24.44de	-	8.88f	-	11.11f
120	-	42.22c	-	20.11de	-	18.89de
128	-	61.11c	-	51.1c	-	36.67c

Same letter in rows represent not significant difference Tukey $p<0.05$

In addition, Table 5 shows that treatments with respect to time are significantly different at 24 hours and 120 hours, with an evident variation in reaction times between each of treatments. In the case of treatments with Tween 80®, they showed similar mortality regardless of the concentration of vinasse applied between 16 and 24 hours. Conversely, a detailed analysis of raw treatments showed that the mortality rates were not significantly different between 96 and 112 hours. However, after 120 hours, the highest mortalities were recorded, with the highest presence of dead individuals at 128 hours. If the concentration in the applications is taken into account, the differences between T100, T75, and T50 treatments are not statistically significant.

In Figure 5, evidence indicates that the addition of the surfactant enhances the efficiency of larval response time to vinasse. As observed, in the untreated treatments, larval mortality occurs at 48 hours, whereas in T + Tween 80®, dead larvae are observed from 8 hours onwards. Furthermore, mortality was higher in the Tween 80® treatment, reaching a maximum of 83.3%. This figure also shows a 88-hour difference in which the first dead individuals begin to be reported, in both cases at a concentration of 100% vinasse with the difference of the surfactant. This type of study provides support for deciding whether it is advantageous to use this enhancer for swift pest control or, conversely, if there is no urgent need requiring the addition of Tween 80®.

Reports by Reyna-Peralta, 2020 [20], show a mortality of *Galleria mellonella* of 93.4% using *Capsicum* oil extract at a concentration of 15 gL^{-1} with a time of 264 hours and a LD50 of 2.78 gL^{-1}. As can be expected, being an extract that contains irritants makes it more effective in its performance, however, vinasse is more efficient at longer application times since the maximum application time was 128 hours.

Moreno-Serrano, 2022 [18] did a study on the application of *Beauveria bassiana* on *Galleria mellonella* larvae. The results were published with a LT_{50} of 175.92 hours, compared to the T50 treatment which was the least efficient, the mean time was less.

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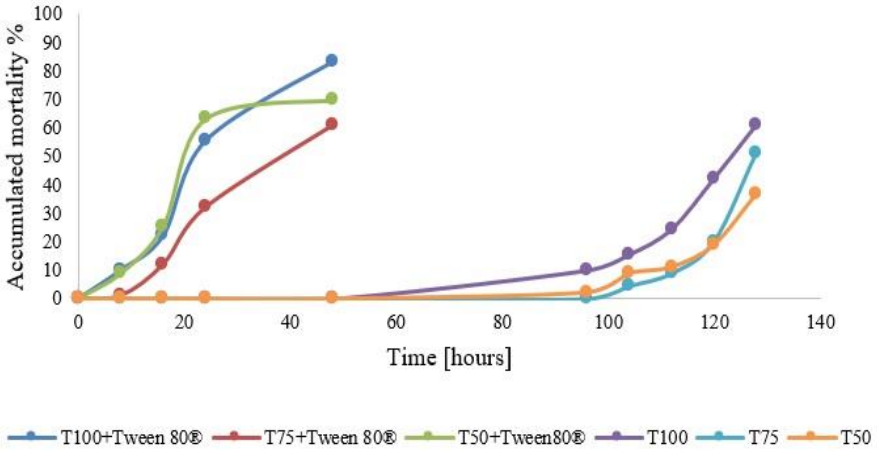

Fig. 5 Effect of different vinasse treatmentes respect to time

It is important to mention that the potential of sotol vinasse as a bioinsecticide is influenced by the addition of surfactant Tween 80® to the different doses in which the bioassay was made, being the LT_{50} variable in which a significant difference is reflected between the time of action of the sotol vinasse. Additionally, the Tween 80® compound added a moisturizing effect to the mixture and allowed a greater adherence of the vinasse on the surface of the cuticle of the insect [14].

Conclusions

The treatments evaluated in this bioassay proved to be efficient, obtaining LD_{50} of up to 46% of vinasse concentration. The difference between treatments is established in the LT_{50} without significant differences between the treatment with Tween 80® and raw vinasse, increasing its efficiency by 82% using surfactant. The bioassay made it possible to establish concentration gradients and times of action of the vinasse against the larvae of *Galleria mellonella*.

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