

Folivory on *Ipomoea murucoides* Roem & Schult exerts metabolic changes related to insecticidal activity against Fall armyworm, *Spodoptera frugiperda* (Walker)

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ABSTRACT

We studied the effects of 4-levels of leaves herbivory (without herbivory, herbivory < 20 %, herbivory > 20 %) and mechanically damaged leaves of *Ipomoea murucoides* Roem. & Schult. We prepared the Hexane, Dichloromethane and Methanol extracts from leaves of these 4-groups and added to meridic diet given to Fall armyworm, [*Spodoptera frugiperda* Walker (Lepidoptera: Noctuidae)] larvae. These extracts chemical composition was analyzed using liquid chromatography coupled to mass spectrometry. The extracts were grouped according to the solvent polarity, however, within each solvent, herbivory treatments were different. The diet containing hexane extract of leaves with herbivory > 20 % and herbivory < 20 % caused maximum mortality (84 % and 68 %, respectively) of *S. frugiperda* larvae. This study showed that mortality of *S. frugiperda* larvae depended on the metabolic chemical compounds variations in the extracts of *I. murucoides*.

Key words: Bio-insecticides, direct plant defence, extract, fall armyworm, folivory, herbivory, insecticidal activity, *Ipomoea murucoides*, metabolomics, non-metric multi-dimensional scaling, *Ogdoecosta biannularis*, *Spodoptera frugiperda*.

INTRODUCTION

Ipomoea murucoides Roem. & Schult. is 2-8 m tall tree, with breast height stem diameter of 40 cm (11,59). It is distributed from Central-western Mexico to Guatemala subtropical and tropical dry forest (11). Within the tropical dry forest, it is found in forest edges and rocky soils in mature forest areas (50). *Ipomoea murucoides* (Figure 1a,b,c) maintains important ecological interactions with arthropods, [insects of the order Coleoptera (Families: Cerambycidae, Buprestidae, Bostrichidae and Elateridae)] who feeds on the phloem and/or xylem sap of *I. murucoides* (57). These trees species also interact with bats [(10), which pollinates the flowers], other plants like epiphytes (55,56), herbs (22) and lianas (18). Its shoots infusions are used to treat the inflammation and also used as firewood (16,32). In addition, *Ipomoea murucoides* is toxic to livestock (33) and is also allelopathic to epiphyte *Tillandsia recurvata* (L.) (55,56). Sesquiterpenes were isolated from the hexane and methanol extracts of *I. murucoides* leaves (53). Its methanol extracts cause the mortality of *Spodoptera frugiperda* Walker (Lepidoptera: Noctuidae) (Figure 2) (58). During the leaf

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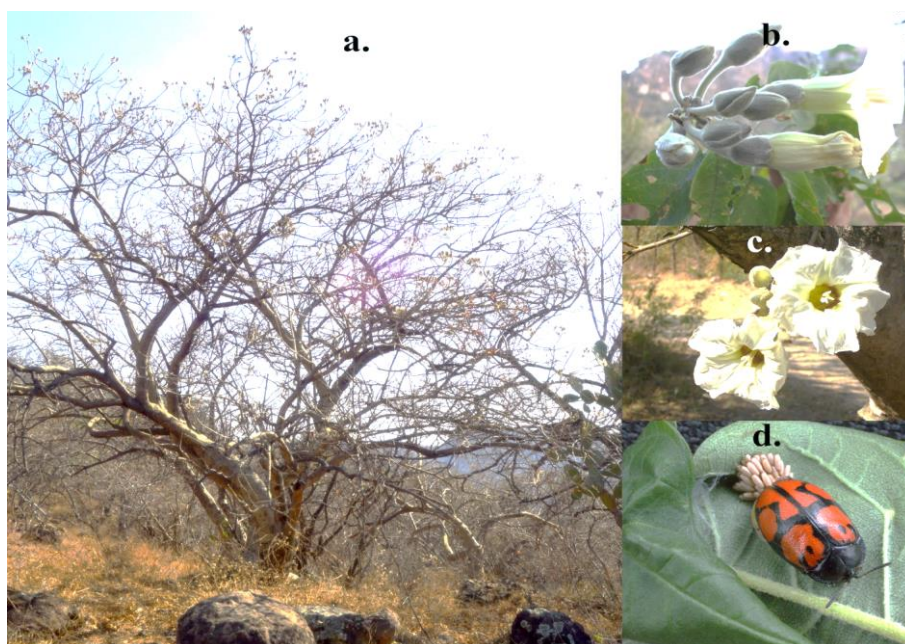


Figure 1. **a.** Fruiting tree of *Ipomoea murucoides*, notice its deciduous phase, **b.** and **c.** White, bell shape flowers of *I. murucoides* and **d.** *Ogdoecosta biannularis* laying eggs in a leaf of *I. murucoides*. (Authorship of Figures 1a, b and d: A. Flores-Palacios. 1c, T. Cruz-Fernández).

production period (May-June), the leaves of *I. murucoides* are mainly eaten by *Ogdoecosta biannularis* (45) (J. E. Smith) (Coleoptera: Chrysomelidae, Figure 1d), causing severe defoliation. Therefore, it is likely that *O. biannularis* induces defence responses in *I. murucoides* related to the insecticidal activity against other insects species, including pests like Fall armyworm, *Spodoptera frugiperda*. Fall armyworm is a holometabolous and polyphagous insect that affects, several crops like soybean and cotton (25). It is distributed, mainly in tropical and subtropical areas (5,12). In México, it is main threat to maize (8), it also attacks different crops in other countries (5,21).

Herbivory causes biochemical changes in plants associated with defence against herbivores, its magnitude produces the metabolic variations in plants. Herbivory induces the synthesis of secondary defence metabolites against herbivores (40,49); thus, plants exhibit high phenotypic plasticity reflected in their metabolome (60,61). Within the same plant species, metabolic variation occurs in response to different intrinsic variables of herbivory such as the type of tissue consumed (49), the species (41), or the developmental stage of herbivore (61). Few studies done in this regard, found that the degree of herbivory (indirectly estimated by herbivore density) modifies the plant metabolome (41). There is evidence that the density of aphid *Brevicoryne brassicae* L. (Homoptera: Aphididae) on leaves of *Brassica nigra* (L.) W.D.J. Koch generates different metabolic patterns (41). *Betula pubescens* subsp. *Czerepanovii* (Orlova) Hämet-Ahti had different metabolic responses depending on the densities of larvae of *Epirritia autumnata* (BKH.) (Lepidoptera: Geometridae) (37). The

different degrees of folivory induce changes in the metabolome and whether these changes could be related to insecticidal activity. The first step to resolve this gap in knowledge is to conduct a fingerprint analysis, which is screening (snapshot) of an organism's metabolome in response to different stimuli, like herbivory. Instead of identifying the metabolites, this analysis shows variations in their patterns. The *Plantago lanceolata* L. metabolic fingerprints varies with the level of specialization of herbivores (51). The fingerprint analysis differs among the phylogenetically related species (Brassicales) and consumed by same herbivore [*Pieris rapae* (L.) (Lepidoptera : Pieridae)] (43).

This study hypothesizes that the metabolites of *I. murucoides* leaves depends on the degree of herbivory and that the extracts from such leaves may have greater insecticidal effects than leaves without herbivory. This study aimed to : (i). determine the patterns of metabolic variations in leaves of *I. murucoides* with different degrees of herbivory by *O. biannularis* and (ii). test if the extracts from leaves with various degrees of herbivory exert variable effects on *S. frugiperda* mortality.

MATERIALS AND METHODS

I. Study Area

The *I. murucoides* plants were collected from the Cerro de la Cruz, San Andrés de la Cal, Tepoztlán, Morelos, Mexico (18°57'22.2"W, 99°06'50.2"N, 1495 m a.s.l.). The study site is located in the "Chichinautzin" Biological Reserve, a protected area of wild flora and fauna. Mean annual precipitation : 1098 mm mean, temperature : 12 to 18 °C (46). The primary vegetation is tropical dry forest, with trees of mean height < 16 m (47). The most abundant trees are *Sapium macrocarpum* Müll. Arg. (Euphorbiaceae), *Bursera copallifera* (DC.) Bullock, *B. fagaroides*(Kunth) Engl., *B. glabrifolia* (Kunth) Engl.(Bursaceae), *Conzattia multiflora* (B.L. Rob.) Standl, *Lysiloma acapulcense* (Kunth) Benth. and *I. murucoides* (59).

II. Leaf collection

In mid June 2018, ten *I. murucoides* trees (4.5 m tall), infested with *O. biannularis* were chosen. From the outer branches of each tree, approx 200 leaves without herbivory (without damage) and 200 leaves with herbivory were collected. According to Romero-Napoles (45), *I. murucoides* produce leaves from the start of rainy season (mid-May) and *O. biannularis* attacks the *I. murucoides* leaves from June to September. Thus, the collected leaves were exposed to herbivory from early June. To compare the herbivory with mechanical damage, before being collected, 200 leaves without herbivory, received 2 perforations (5 mm each) with paper puncher. These leaves were left on each tree for 2.0 h and then collected. All the collected leaves were dried at ambient temperature and pressed between pages of newspaper and cardboard to keep the leaf blades expanded. Each leaf was scanned (Epson scanner 1-210) to determine its leaf area with the program Sigma ScanPro (Systat Software Inc.).

To estimate the herbivory (%) in the collected leaves, three linear regression models were constructed using data from the leaves without herbivory (with intact blade). In these models, the dependent variable was the logarithm of the leaf area, while the independent variables for each model were as under:

- (a) Logarithm of Length ($y = -0.6184 + 1.9282_{\log \text{ length}}$, $F_{1, 1925} = 10270$, $P < 0.001$, $R^2 = 0.84$),
 (b) Logarithm of Width ($y = 0.4935 + 1.6273_{\log \text{ width}}$, $F_{1, 1925} = 14886$, $P < 0.001$, $R^2 = 0.88$) and
 (c) Logarithms of Length and Width ($y = -0.1036 + 1.0005_{\log \text{ width}} + 0.9015_{\log \text{ length}}$, $F_{1, 1925} = 2669.7$, $P < 0.001$, $R^2 = 0.93$).

When only the length was known, the model (a) was used to estimate the area of consumed leaves, the (b) was used when the width was known and the (c) was used when both length and width were known. Based on these measurements, the herbivory (%) per leaf was calculated as the ratio between the leaf's measured and estimated area values.

Due to the high variations in the herbivory area (Figure 3), and to obtain an adequate tissue sample, leaves with herbivory were categorized into 4 Groups: (i). Without herbivory, (ii). with herbivory < 20 %, (iii). with herbivory > 20 % and (iv). leaves with mechanical damage. The 20 % herbivory limit was considered, because the number of categories did not modify the trend of positive asymmetry (coefficient of asymmetry = 1.78).

III. Plant extracts

To obtain enough extract volumes for the experiments, the leaves of each category were mixed regardless of tree identity; they were milled in an electric liquidizer (Osterizer 4655, 4655-013). Although the measure of variability among individuals was lost, this variability lacks biological interpretation (individuals are random levels of a random variable and differences among individuals are impossible to interpret), due to the presence of confounding factors (13). At the same time, the pooled extracts represent the general properties of *I. murucoides* for each herbivory treatment regardless of the individual identity.

In August 2018, each leaf sample was macerated at ambient temperature and extracted with solvents of different polarities (Hexane, Dichloromethane and Methanol). Extraction was done for 72 h, thrice consecutively, with each solvent. The extracts were concentrated under vacuum (BÜCHI R-100), the hexane and dichloromethane at 39-40 °C, while the methanol extracts were concentrated at 49-50 °C. The 12 extracts (3-organic extracts: Hexane, Dichloromethane and Methanol × 4-levels of herbivory: (Without herbivory, herbivory < 20 %, herbivory > 20 % and mechanic damage) were stored at 5 °C in glass jars wrapped in aluminium paper until subsequent processing. Each of the 12 extracts was considered a treatment.

IV. *Spodoptera frugiperda* survival assays

Individuals of *S. frugiperda* were obtained from a colony reared since 2004 in a growing chamber (REVCO Scientific Inc., BOD50ABA) under temperature, relative humidity, and photoperiod regimes of 27 °C ± 1.5 °C, 60 % ± 5 % and 16 h light and 8 h darkness, respectively. From June 2018 to February 2019, survival assays were done at the Laboratorio de Control Biológico, Centro de Investigación en Biotecnología at Universidad Autónoma del Estado de Morelos (Figure 2).

To determine whether the magnitude of herbivory degree (without herbivory, herbivory < 20 %, herbivory > 20 %, and mechanic damage) and/or type of extract (Hexane, dichloromethane and methanol) affects the survival of *S. frugiperda*, a modified meridic diet (59) was prepared and offered to neonate larvae of *S. frugiperda*. 1.5 mL of meridic diet mixed with the extract (2 mg/mL) was placed in each plastic dish (2.8 cm dia) used in all 12

treatments. To avoid the fungal infection, the Petri dishes were cleaned with 70 % alcohol. There were two controls; (i). Meridic diet without extracts and the (ii). meridic diet with a broad-spectrum insecticide PIQ2 (2000 mg/L, Green Corp. biorganiks de Mexico, S. A. de C.V.). As the extracts were insoluble in water, we used TWEEN 20 (SIGMA Cell Cultured Tested P228 7-100 ml). We had done a previous experiment, comparing the effects of TWEEN (1 %, v/v) and a control (meridic diet) on the survival time of *S. frugiperda*. The results showed similar statistical effects ($t = -1.83$, $d.f. = 298$, $P = 0.066$; 21.08 ± 4.71 days in the control vs. 19.56 ± 6.59 days in tween). The experimental unit was each plastic dish with one neonate larva of *S. frugiperda* (25 plastic dishes per treatment and controls) were laid in the Growth Chamber with the same conditions explained before.



Figure 2. Left : *Spodoptera frugiperda* larva in a Petri dish with meridic diet and Right : *S. frugiperda* pupa image. (Photos D. Ocampo-Antonio).

Food availability was checked daily, and in case of total consumption, the same amount of meridic diet was added again. After 7-days of starting the experiment, individuals were transferred to Petri dishes maintaining the same environmental conditions, and the food was withdrawn when pupation began. Cotton moistened with 5 ml distilled water was placed in each Petri dish to maintain the humidity inside. The cotton was replaced every other day to avoid dryness. The number of living individuals was recorded till 23 days.

V. Metabolic analysis

An untargeted study was done to detect the possible metabolic patterns among treatments. Extracts were analyzed by liquid chromatography (Agilent 1290, Infinity II, Singapore) coupled to quadrupole time of flight mass analyzer (QTOF, Agilent G6545, Germany) with Dual Jet Stream ionization source. Samples were prepared 1 mg/mL in methanol (MS grade), sonicated 10 min, and filtered 0.2 μm before analysis. Chromatography was run with a zorbax eclipse plus C18 2.1 \times 100 mm, 1.8-micron column at 20°C, injection volume was 9 μL , H₂O (solvent A) - MeOH (solvent B) both with 0.1 formic acid gradient at flow 0.3 mL/min were used as mobile phase as follows: 85 % A 10 min, change to 100 % B on 3 min and kept until minute 14, after that, flow returned to original conditions for 0.5 min previous next injection. Positive mode ESI with a range of masses of 100-3000 m/z was used on the QTOF with 3500 and 140 V for VCap and fragmentor, respectively. Data were processed on Mass Hunter Qualitative analysis B.07.00 software; compounds were extracted using an isotopic model for common organic molecules for peaks with at least 1.5 % of the relative height and then identified using Metlin Metabolites AM database (http://metlin.scripps.edu/landing_page.php?pgcontent=main Page) by comparing the mass and fragmentation pattern. Within each treatment, mass, retention time, and fragmentation pattern were used to identify each component.

VI. Statistical Analyses

A Cox proportional hazard analysis (28) was done to find the effects of herbivory degree and type of extract on mortality of *S. frugiperda*. Data from survival of *S. frugiperda* under insecticide and diet alone were also included. The analyses were done in R version 3.4.3. (41) with libraries survival (54), survminer (27) and multcomp (20).

An ordination analysis of the 12 extracts was done; a matrix of presence/absence of Jacquard similarity index was constructed considering the chemical composition of each extract. Based on this matrix, a non-metric multidimensional scaling (NMDS) (24) analysis was conducted for detecting ordination patterns among extracts. In this analysis, the retention times of the chemical compounds of treatments were used as variables. After, the coordinates of NMDS dimensions were correlated with retention times of each of the chemical components of each treatment. Correlation coefficients were significant at $P < 0.05$. NMDS analysis was performed with the library vegan 2.5-6 (35).

RESULTS AND DISCUSSION

A total of 1900 leaves without herbivory and/or damaged by herbivores were collected. Of the leaves with herbivory, the mean lost area was 13.94 ± 12.49 %; the leaf least affected by herbivory had < 0.01 % of lost area while, the maximum was 89.39 %. Of the total leaves with herbivory, 78.85 % (1294 leaves) had less than 20 % of herbivory, while 21.15 % (348 leaves) of the leaves had herbivory damage > 20 % (Figure 3).

I. Survival analysis

We found that the type of extract ($\chi^2 = 17.31$, $d.f. = 2$, $P < 0.001$), the herbivory degree ($\chi^2 = 38.38$, $d.f. = 5$, $P < 0.0001$) and their interaction ($\chi^2 = 23.31$, $d.f. = 10$, $P < 0.01$) affected the survival of *S. frugiperda*. Previous evidence of allelopathic and insecticidal properties of *I. murucoides* shows that dichloromethane bark extract inhibits the seeds

germination (up to 58 %) of epiphyte *T. recurvata* (55,56), and methanol leaves extract causes 50 % deaths of *S. frugiperda* larvae (58).

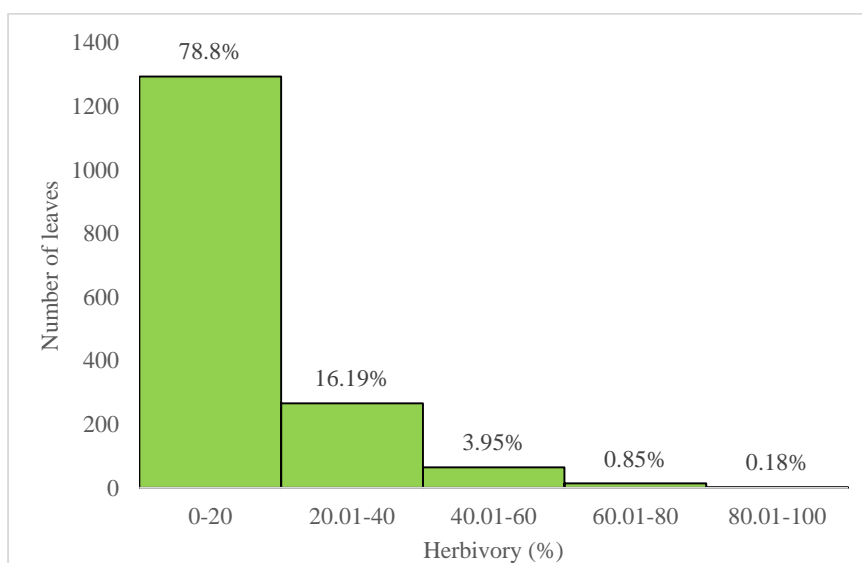


Figure 3. Herbivory categories in 1641 leaves of *Ipomoea murucoides*.

Unlike other studies, where the methanol extract had insecticidal properties (56); here, we found that hexane extract caused the fastest and highest mortality ($P = 0.0003$) of *S. frugiperda* larvae (52.4 %). However, both, methanol (46.2 %) and dichloromethane extract (37.3 %, Figure 4) were less harmful ($P > 0.05$). The differences between our results and that of Vera-Curzio *et al.* (58) may be due to the extraction technique used. We did extraction using consecutively 3-solvents of increasing polarity, whereas, Vera-Curzio *et al.* (58) did extractions directly with methanol. It is known that hexane mainly extract fats, waxes, mono and sesquiterpenes. Chemicals obtained with dichloromethane include intermediate polarity compounds like di and triterpenes; meanwhile, methanol extract contains polar metabolites like flavonoids, tannins, or glycosides (6,36). Besides, the methanol also extracted the growth inhibitors [e.g. tricolorin A, (3)] glycosidic resins found in the Convolvulaceae family (11). The hexane extracted greater diversity of metabolites than methanol; these metabolites may interact synergistically, resulting in greater insecticidal effects than methanol extract.

It is known that *I. murucoides* has insecticidal and allelopathic properties (30,55,56,58), but there were no former studies with this specie, related to the herbivory degree with its insecticidal activity and with its metabolome variations. The herbivory treatments, as well as the insecticide, caused greater larvae mortalities (Tukey Test, $P < 0.05$) than control (meridic diet, 16 % mortality). Regardless of the type of extract, herbivory treatments and insecticide had similar effects on larvae mortalities of *S. frugiperda* (Tukey Test, $P > 0.05$); 60 % (herbivory > 20 %), 54.7 % (insecticide), 48 % (without herbivory), 45.3 % (herbivory < 20 %) and 40 % (mechanical damage).

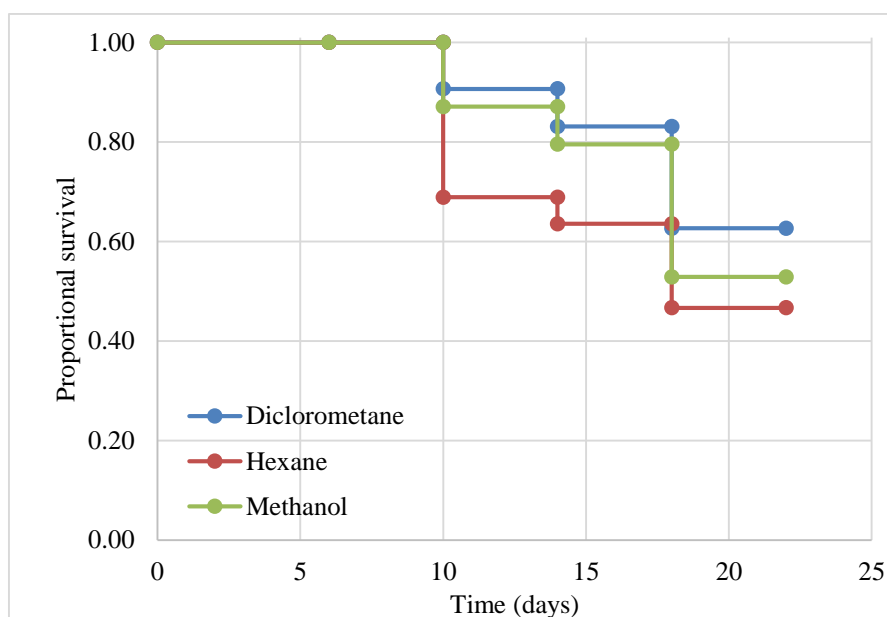


Figure 4. Kaplan-Meier curves showing the effects of Hexane, Dichloromethane and Methanol extracts from *Ipomoea murucoides* leaves on survival of *Spodoptera frugiperda* insects

We expected that herbivory made by *O. biannularis* on *I. murucoides* leaves would be reflected in the insect mortality, probably due to metabolic changes. Herbivory elicits chemical reactions, whereas, the jasmonates (JA, MeJA) play an important role in plant defence (2). A possible explanation of the same effects exerted by different herbivory degrees is that as we collected unwounded and wounded leaves from the same individuals, it is probable that they were vascularly connected, and molecules related to plant defence travel from damage to undamaged tissues. The polypeptide systemin is released in response to herbivory. It acts distally by moving through the phloem and is detected by receptors in the plasma membrane, the linolenic acid increases and acts as the precursor of jasmonic acid (JA). JA induces the synthesis of defence proteins or secondary metabolites (phenols, terpenoids, phenylpropanoids etc.), activating the acquired systemic resistance (52,63).

The herbivory stimulates the release of volatile organic compounds (VOCs) (7,14,19) like methyl jasmonate (MeJA), or other molecules of low molecular weight (fatty acids derivatives, alcohols, aromatic compounds, aldehydes, mono and sesquiterpenes). These airborne molecules can act as infochemicals by "alerting" the undamaged parts within the same individual (49) or other nearby plants to activate their defence (26). This kind of communication could have occurred within and among the sampled trees of *I. murucoides*, so that no differences were observed among herbivory treatments on the mortality of *S. frugiperda*. Finally, it is possible that any other biotic (pathogens, plant-plant interactions) and abiotic factors (drought stress, UV exposure), plus herbivory, make the plant interaction more complex (1,17,34) and this would be reflected in the metabolome of *I. murucoides* leaves.

The interactions between type of extract \times herbivory degree followed the similar pattern to main factors. The mortality rates caused by the hexane-herbivory $> 20\%$ and hexane-herbivory $< 20\%$ extracts on *S. frugiperda* larvae were the highest in this study with 84 % and 68 %, respectively (Table 1). But were also the highest than other studies, where methanolic extract of *I. murucoides* caused mortalities of 46.16 % for larvae and 59.6 % for individuals of 5th instar (58). Besides two isolated murucoidins (XIX and XX) from bark extracts of *I. murucoides* did not cause mortality in *S. frugiperda* (30). On the other hand, dichloromethane extracts of any herbivory treatment had the lowest effects on larvae mortality (Table 1).

Table 1. Effects of different solvents extracts of *I. murucoides* leaves on mortality (%) of *S. frugiperda* larvae.

Treatment	Solvents extracts mortality (%)		
	Hexane	Dichloromethane	Methanol
Without herbivory	40(21-61) ^{bc}	36(18-57) ^{bc}	60(39-79) ^{ab}
Herbivory $< 20\%$	68(46-85) ^{bc}	40(21-61) ^{bc}	56(35-76) ^{ab}
Herbivory $> 20\%$	84(64-95) ^a	36(18-57) ^{bc}	60(39-79) ^{ab}
Mechanical damage	48(28-69) ^{abc}	20(7-41) ^{bc}	52(31-72) ^{abc}
Insecticide	54(44-64) ^b	47(37-57) ^b	43(33-53) ^{abc}
Meridic diet	16(5-36) ^c	16(5-36) ^c	16(5-36) ^c

Extracts have three different polarities and come from leaves with different degree of herbivory. Different letters indicate significant difference between the proportions of mortality ($P < 0.05$). In parenthesis are the 95 % confidence intervals for binomial variables.

Like the crude hexane extract, the greatest mortalities were caused by the extract hexane-herbivory $> 20\%$ had major diversity of metabolites than those found in other interactions of extract-herbivory degree. Then the probability of finding the molecules with insecticidal properties increases.

II. Metabolic analysis

One of the objectives of metabolomic research in plants is to search for patterns that indicate changes in the metabolome in response to environmental stimuli (1,34), whether, abiotic (e.g. salinity, pH) or biotic (e.g. herbivory). Despite the importance of herbivory in the structure of plant communities in both natural and agricultural ecosystems, few metabolic

Table 2. Number and metabolites (%) detected by LC-MS only in four main grades of herbivory.

Herbivory	No. of metabolites (%)
WH [Without herbivory (Control)]	21 (16.93)
Herbivory $< 20\%$	22 (17.74)
Herbivory $> 20\%$	37 (29.83)
MD (Mechanical Damage)	25 (20.16)
WH-MD	1 (0.8)
Herbivory < 20 -MD	4 (3.22)
WH- Herbivory $< 20\%$ - Herbivory $> 20\%$ - MD	14 (11.29)

WH : without herbivory, MD : mechanical damage. The number of metabolites shared among intersections of herbivory degrees is also shown. Treatments combinations whose shared metabolites was zero were not shown.

studies have been done (40,44,48). In this study, we detected 124 metabolites distributed in the 12 extracts of *I. muruoides* leaves (Supplementary material). Hexane extracts contained 41.13 % of the metabolites, dichloromethane and methanol extracts had 23.38 %. The rest metabolites were shared among extracts (Figure 5). On the other hand, there were more metabolites, when herbivory was more than 20 % (Table 2).

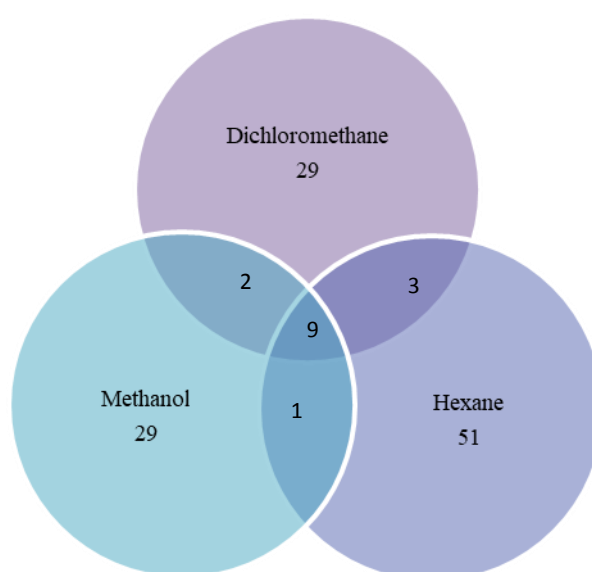


Figure 5. Distribution of metabolites in 3-extracts (Hexane, Dichloromethane and Methanol) of *I. muruoides* leaves.

Extracts were mainly separated according to the solvent polarity (stress = 0.11). The first dimension showed similarity among methanol extracts, and these were separated from dichloromethane and hexane extracts and were similar to dichloromethane/ mechanical damage (Table 3, Figure 6). The extracts did not follow a clear grouping pattern in dimension 2. The positive area of dimension 2 tend to join those extracts with mechanical damage and with herbivory > 20 %; meanwhile, it seems that extracts without herbivory and with herbivory < 20 % were positioned in the negative area of the same dimension (Figure 6).

The metabolome changes occur in response to the density of herbivores (37,41). Unlike previous studies, we directly measured the foliar area consumed in leaves of *I. muruoides*, thus metabolic changes could be mainly attributed to influence the herbivory by *O. biannularis*; however, extracts did not ordinate according to the herbivory level but by metabolic similarities, following the extract polarities. NMDS-ordination separated the methanol from hexane extracts, while, dichloromethane extracts shared chemical compounds with both.

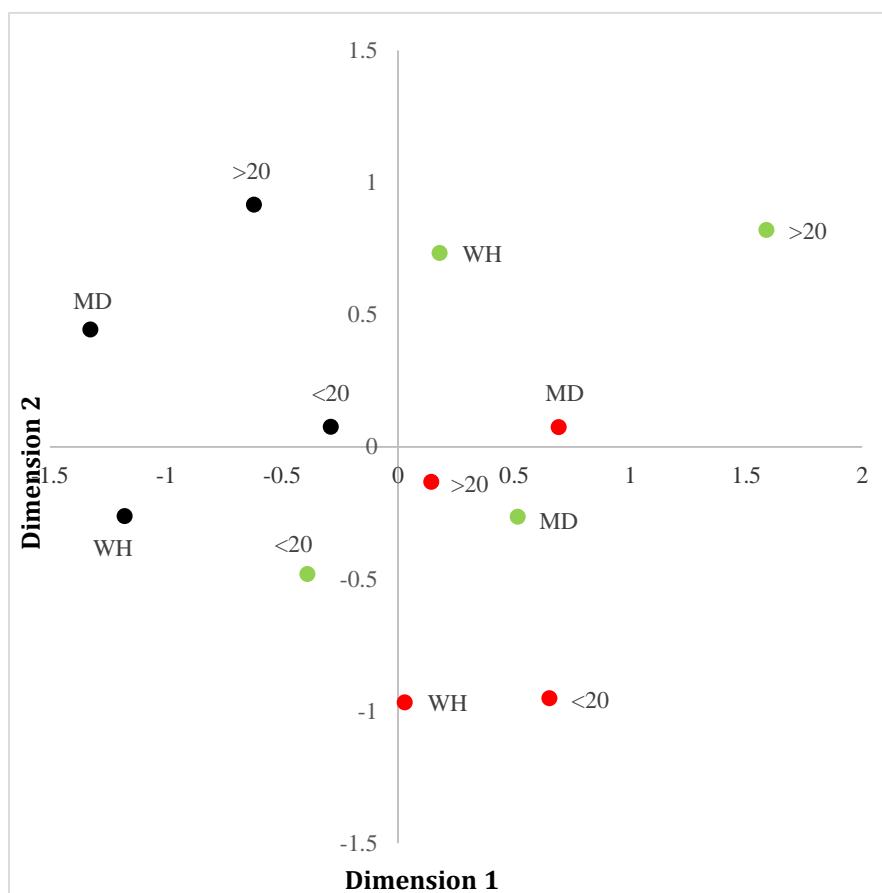


Figure 6. Ordination of 12 extracts from *Ipomoea murucoides* leaves.

Ordination was based on the distance matrix of Jacquard similarity of retention times of chemical compounds detected in 12 extracts. Black circles refer to Methanol extracts, green circles to Dichloromethane extracts and red circles to Hexane extracts. WH = Without herbivory, < 20 % = Herbivory < 20 %, > 20 = Herbivory > 20 % and MD = Mechanical damage.

Without considering the polarity of the extracts, results did not show any effects of herbivory on the metabolome; this could be attributable to the lack of biological and technical replicates. In this study, the plant material of each treatment was combined because their mixture would represent the metabolic changes at population level of *I. murucoides*. However, if measurements had been done at the individual level, keeping the identity of each sample would provide a more precise metabolic variation in response to herbivory. Another reason is that, before collection, leaves were exposed to herbivory for at least 15 days; then, as was mentioned before, plants could have acquired systemic resistance, uniformizing their metabolome.

Table 3. Diversity in the classic Jacquard similarity index among 12 extracts (3 solvents × 4 herbivory degrees) of *L. mimnicoides* leaves.

	H<20	H=20	H>20	H<20	H=20	H>20	D<20	D=20	D>20	M<20	M=20	M>20	M<MD	M=MD	M>MD
H<20	0.174	1	0.208	0.136	0.107	0.174	0.026	0.185	0.095	0.174	0.074	0.077			
H=20	0.208	0.214	1	0.231	0.226	0.259	0.119	0.3	0.154	0.161	0.094				
H>20	0.136	0.154	0.231	1	0.133	0.154	0.103	0.207	0.13	0.2	0.103	0.069			
D<20	0.107	0.125	0.226	0.133	1	0.161	0.114	0.171	0.067	0.241	0.188	0.088			
D=20	0.174	0.143	0.259	0.154	0.161	1	0.071	0.194	0.167	0.231	0.097	0.138			
D>20	0.026	0.071	0.119	0.103	0.114	0.071	1	0.136	0.025	0.071	0.068	0.022			
M<20	0.095	0.077	0.154	0.13	0.067	0.167	0.025	0.1	1	0.273	0.083	0.027			
M=20	0.174	0.143	0.308	0.2	0.241	0.231	0.071	0.233	0.273	1	0.259	0.179			
M>20	0.074	0.063	0.161	0.103	0.188	0.097	0.068	0.083	0.154	0.259	1	0.25			
M<MD	0.077	0.031	0.094	0.069	0.088	0.138	0.022	0.027	0.208	0.179	0.25	1			

H=Hexane, D=Dichloromethane, M=Methanol, WH=Without herbivory, < 20 % = Herbivory < 20 %, > 20 % = Herbivory > 20 %, MD=Mechanical damage.

Table 4. Chemical compounds that correlate significantly ($P > 0.05$) with coordinates of dimensions (D) 1 and 2 calculated in NMDS analysis.

Metabolite	D1	D2	r	Hexane			Dichloromethane			Methanol					
				WH	<20	>20	MD	WH	<20	>20	MD	WH	<20	>20	MD
cis-9,10-epoxy stearic acid	✓		0.64	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Terpenyl isovalerate	✓		0.61	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
(+)-Eudesmin	✓		-0.77	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
C16 Sphinganine	✓		-0.71	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Artemisyl propionate	✓		-0.79	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Corrinoid	✓		-0.70	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
5β-Pregnane-3α,17α,20α-triol-11-one	✓		0.59	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
17-Hydroxy-3-oxo-19-nor-5α,17α-pregnane-21-carboxylic acid, γ-lactone	✓		-0.71	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
*Unidentified metabolites	✓		0.61	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Bovigunone 4	✓		0.61	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Compactin diol lactone	✓		0.61	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Doisynoestrol	✓		0.61	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
DG(22:2(13Z,16Z)/14:1(9Z)/0:0)	✓		-0.88	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
MG(0:0;16:0;0:0)	✓		0.68	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
Iriotonolide 1a	✓		0.61	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
(4S,5S)-(+)-Germacrene 4,5-epoxide	✓		-0.78	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓

There are shown Pearson coefficient of correlation (r) and the extracts in which compounds were detected by LC-MS. ✓ means presence. *Unidentified metabolites (mass@retention time) all of them are only present in dichloromethane extract >20 and present r=0.061, Cpd 1: 172.0716@0.844, Cpd 2: 168.1128@4.340, Cpd 3: 678.5044@4.484, Cpd 5: 604.4190@5.498, Cpd 6: 712.4380@5.599, Cpd 7: 276.1345@5.719, Cpd 10: 287.2829@6.166, Cpd 14: 1372.9062@6.951, Cpd 22: 604.4161@7.272, Cpd 30: 550.3493@8.121, Cpd 33: 700.4905@8.834, Cpd 36: 510.4428@9.801, Cpd 41: 920.664@10.405, Cpd 47: 591.4502@11.038, Cpd 50: 1008.6942@11.273, Cpd 52: 2715.7785@11.982, Cpd 53: 910.6211@12.521, Cpd 55: 337.3346@13.211, Cpd 57: 131.9511@15.255.

There were 34 metabolites associated significantly ($P < 0.05$) with one of the two dimensions; 25 of them were correlated positively with dimension 1 (Table 4, including unidentified metabolites). Five metabolites [(+)-Eudesmin, C16 Sphinganine, Artemisyl propionate corrinoid and 17-Hydroxy-3-oxo-19-nor-5 α , 17 α -pregnane-21-carboxylic acid, gamma- lactone] were correlated negatively with dimension 1 and had a $r \leq -0.70$ (Table 4). Four metabolites were significantly correlated with dimension 2 ($P < 0.05$), two of them were positively associated, meanwhile, the other two were negatively associated (Table 3). Those metabolites were correlated negatively with dimension 2 had a $r \leq -0.7$ [DG(22:2(13Z,16Z)/14:1(9Z)/0:0) and (4S,5S)-(+)-Germacrone 4,5-epoxide, Table 4].

Hexane and dichloromethane extracts had two metabolites implicated in chemical plant defence that correlated significantly with the first dimension: cis-9,10-epoxy stearic acid (C₁₈H₃₄O₃, Figure 7a) and (terpenyl isovalerate (C₁₅H₂₆O₂, Figure 7b). The first metabolite is an oxylipin (oxygenated fatty acids) that could be involved in plant defence as part of cutine or as phytoalexins (15), moreover, it was also present in methanol extract, the second most bioactive extract in this study. Meanwhile, terpenyl isovalerate is a monoterpene currently used as a repellent against insects (patent US2019047937 (23,29).

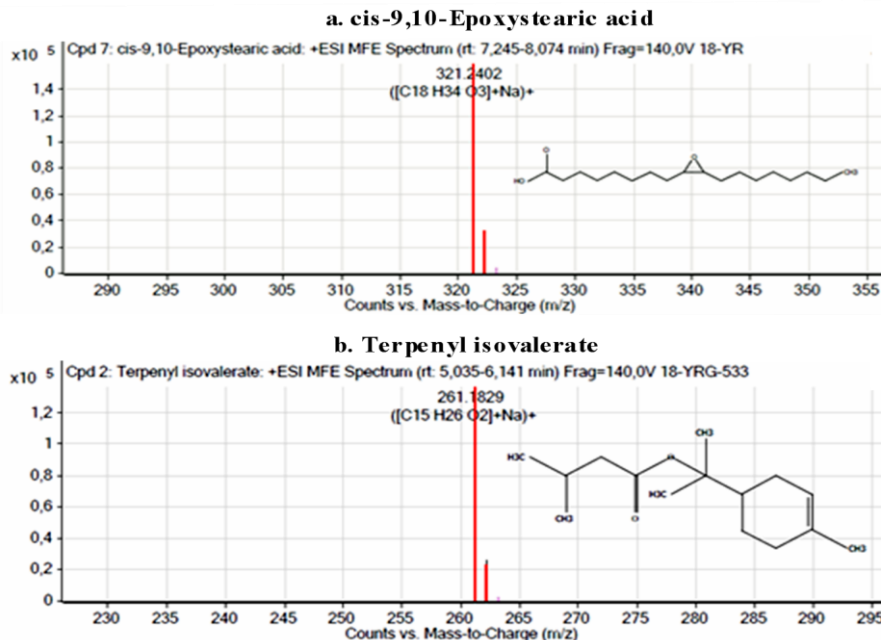


Figure 7. MFE MS zoomed spectrum of **a.** cis-9,10-epoxy stearic acid and **b.** Terpenyl isovalerate.

Two metabolites found in hexane and dichloromethane extracts maintained a negative correlation with dimension 1; corrinoid (cobalt-containing compound) which is produced by prokaryotes (4,39); probably, the corrinoid detected in leaves of *I. muruoides* could be the result of endophytic plant bacteria. The other metabolite was C16 Sphinganine (C₁₆H₃₅NO₂), a type of sphingolipid, that is part of the eukaryotic membrane and is involved

in plant defence against pathogens (9,31). It is possible that leaves of *I. muruoides* did not exclusively face herbivory, but pathogens infestation could be other biotic factors affecting these trees. The methanol extract also contained the lignan (+)-Eudesmin (C₂₂H₂₆O₆), which has antifungal properties (38).

CONCLUSIONS

We found grouping patterns of *I. muruoides* extracts according to their polarity. The herbivory degree by itself did not affect the metabolome of this specie. However, the effects of hexane/herbivory >20 % and hexane/herbivory > 20 % extracts on the mortality of *S. frugiperda* were the highest. Cis-9,10-epoxy stearic acid and terpenyl isovalerate were metabolites that may be related to *S. frugiperda* mortality; however, the entire mixture of compounds contained in this extract should also be considered for future research. This metabolic fingerprint approach broadens our understanding of the response of plants in their interaction with insects and specifically of *I. muruoides* with *O. biannularis*. The information generated can serve as a basis to control and reduce populations of pest insects like *S. frugiperda*.

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DECLARATION

We declare that all authors of this Ms have made substantial contributions. We have not excluded any author that substantially contributed to this Ms. We have followed our ethical norms established by our respective institutions.

CONFLICT OF INTEREST

The authors announce that they have no conflict of interest.

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