

CHEMICAL PROFILING OF THE URTICATING TRICHOMES FROM *Cnidoscolus multilobus* (“MALA MUJER”) AND THEIR ANTIMICROBIAL ACTIVITY

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ABSTRACT: *Cnidoscolus multilobus* (Pax.) I.M. Johnst (Euphorbiaceae) is a stinging plant widely distributed in the south of Mexico. Currently, chemical and biological properties of the endogenous fluid from the prickles of *C. multilobus* are still unknown. Thus, the objective of this work was to identify the inflammatory-inducing toxins contained in the prickles of *C. multilobus* and their possible antimicrobial activity. The chemical identification and quantification of these toxins were carried out by GC-MS and HPLC-DAD. Simultaneously, the endogenous fluid and its most abundant constituents (commercially acquired) were individually tested against opportunistic phytopathogens using the broth microdilution method and XTT antifungal method. According to our results, the fluid contained histamine (17.3-28.4 $\mu\text{g mL}^{-1}$), dimethylethylamine (1.7-3.6 $\mu\text{g mL}^{-1}$), acetylcholine (0.6-1.3 $\mu\text{g mL}^{-1}$), serotonin (24.6-45.8 $\mu\text{g mL}^{-1}$), oxalic acid (2.2-9.6 $\mu\text{g mL}^{-1}$) and formic acid (0.9-4.7 $\mu\text{g mL}^{-1}$). The *in vitro* growth of *Pseudomonas syringae* pv. tabaci TBR2004 (MIC, 122.2 $\mu\text{L mL}^{-1}$), *P. syringae* pv. tomato DC3000 (MIC, 105.8 $\mu\text{L mL}^{-1}$), *Fusarium oxysporum* ATCC 62506 (MIC, 97.4 $\mu\text{L mL}^{-1}$) and *Phytophthora capsici* ATCC 15399 (MIC, 163.2 $\mu\text{L mL}^{-1}$) was inhibited by the fluid. Contrarily, the natural fluid had no effect on the *in vitro* growth of *Clavibacter michiganensis* AB299158. The individual assessment of the main compounds dissolved in the fluid revealed that histamine possesses substantial anti-phytopathogenic activity (145.1-219.5 $\mu\text{L mL}^{-1}$). According to our results, pain-inducing toxins from the prickles of *C. multilobus* not only are toxic for mammal species, these substances also exhibit inhibitory properties against opportunistic phytopathogens.

Key words: *Cnidoscolus multilobus*, endogenous fluid, histamine, serotonin, organic acids, antimicrobial.

RESUMEN: *Cnidoscolus multilobus* (Pax.) I.M. Johnst (Euphorbiaceae) es una planta con pelos urticantes ampliamente distribuida en el sur de México. En la actualidad, las propiedades químicas y biológicas del fluido endógeno de las espinas de *C. multilobus* se desconocen. De este modo, el objetivo de este trabajo fue identificar los compuestos tóxicos presentes en el fluido de los tricomas de *Cnidoscolus multilobus* y su posible actividad antimicrobiana. La determinación y cuantificación de las sustancias tóxicas se efectuó por GC-MS y HPLC-DAD. Simultáneamente, el fluido endógeno y sus compuestos más abundantes (adquiridos comercialmente), fueron individualmente ensayados contra fitopatógenos oportunistas mediante el método de microdilución en caldo con rezasurina y el método XTT anti-fúngico. De acuerdo a nuestros resultados, el fluido contuvo histamina (17.3-28.4 $\mu\text{g mL}^{-1}$), dimetiletilamina (1.7-3.6 $\mu\text{g mL}^{-1}$), acetilcolina (0.6-1.3 $\mu\text{g mL}^{-1}$), serotonina (24.6-45.8 $\mu\text{g mL}^{-1}$), ácido oxálico (2.2-9.6 $\mu\text{g mL}^{-1}$) y ácido fórmico (0.9-4.7 $\mu\text{g mL}^{-1}$). El crecimiento *in vitro* de *Pseudomonas syringae* pv. tabaci TBR2004 (MIC, 122.2 $\mu\text{L mL}^{-1}$), *P. syringae* pv. tomato DC3000 (MIC, 105.8 $\mu\text{L mL}^{-1}$), *Fusarium oxysporum* ATCC 62506 (MIC, 97.4 $\mu\text{L mL}^{-1}$) y *Phytophthora capsici* ATCC 15399 (MIC, 163.2 $\mu\text{L mL}^{-1}$) fue inhibido por el fluido natural. Contrariamente, el fluido natural no tuvo efecto sobre el crecimiento *in vitro* de *Clavibacter michiganensis* AB299158. El ensayo individual de los principales compuestos disueltos en el fluido reveló que la histamina posee una actividad antimicrobiana sustancial (145.1-219.5 $\mu\text{L mL}^{-1}$). De acuerdo con nuestros resultados, las toxinas productoras de dolor en las espinas de *C. multilobus* no son dañinas solamente para especies de mamíferos, estas también exhiben propiedades inhibitorias contra fitopatógenos oportunistas.

Palabras clave: *Cnidoscolus multilobus*, fluido endógeno, histamina, serotonina, ácidos orgánicos, antimicrobianos.

INTRODUCTION

The genus *Cnidoscolus* comprises about 50 species spread around the world (Martínez *et al.*, 2002). Approximately, 20 species are categorized as endemic plants of Mexico and are widely distributed in tropical and subtropical zones from the states of Oaxaca, Puebla, Veracruz and San Luis Potosí (Martínez *et al.*, 2002; Steinmann, 2002). *C. urens*, *C. aconitifolius*, *C. chayamansa* and *C. multilobus* are frequently used as local medicines (Lookadoo and Pollard, 1991; Martínez *et al.*, 2002; Steinmann, 2002; Rosas-Piñón *et al.*, 2012). In these states, *C. multilobus* is known as "mala mujer" because of the severe irritation and itching caused by its urticating hairs (fig. 1).

According to some scientific reports, the endogenous fluid of the prickles from *C. oligandrus* and *C. texanus* contains diverse inflammatory inducing compounds including biogenic amines and organic acids (Cordeior *et al.*, 1983; Lookadoo and Pollard, 1991). This property confers to the *Cnidoscolus* genus an efficient chemical defense against mammal herbivore species. In addition, the leaf infusion of *C. multilobus* is used in traditional medicine to treat toothache, hemorrhage, arthritis, vaginal infections and to induce breast milk in breastfeeding women (Rosas-Piñón *et al.*, 2012, Jiménez-Arellanes *et al.* 2014). On the other hand, the industrial use of *C. multilobus* is suggested as an inexpensive coagulant to produce traditional cheeses since the crude sap contains substances able to curdle the casein in milk (Lagunes-Olivares *et al.*, 2017). Currently, only the investigations performed by Delgado *et al.* (1994) and Rosas-Piñón *et al.* (2012) describe some natural products and biological activities of *C. multilobus*. From the latter studies, bioactive triterpenes such as moretenyl acetate, moretenone, lupeil acetate, β -sitosterol, β -amyryn and oleanolic acid were identified and isolated. Interestingly, the evaluation of the aqueous fraction of *C. multilobus* shows a substantial antibacterial activity against oral pathogenic bacteria such as *Streptococcus mutans* and *Porphyromonas gingivalis* (Rosas-Piñón *et al.*, 2012). To the best of our knowledge, there is not any study focused on the chemical composition of the endogenous fluid from the prickles of *C. multilobus*, neither on the possible ecological role of this fluid against phytopathogens. Considering this fact, we focused on the chemical characterization of the natural fluid isolated from the stinging hairs of this plant and in the possible role of such fluid as an anti-phytopathogenic agent.

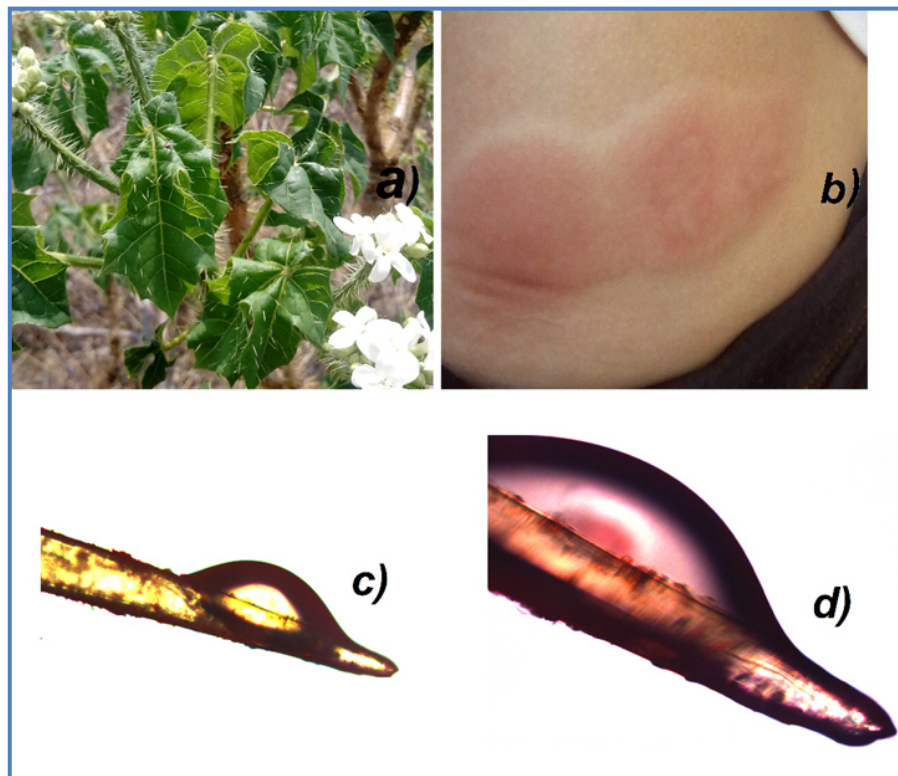


Fig. 1. *Cnidocolus multilobus* grown under field conditions. B) Urticant effects of *Cnidocolus multilobus* on human skin. C) Close up view of the stinging prickles of *Cnidocolus multilobus* at 20X. D) Close up view of the stinging prickles of *Cnidocolus multilobus* at 60X. The endogenous fluid at the top of the prickle is shown.

MATERIAL AND METHODS

Plant material and extraction

In order to determine qualitative and quantitative differences in chemical content, *Cnidocolus multilobus* was collected in Miahuatlán de Porfirio Díaz, Oaxaca (16° 32' 58''N; -96° 54' 48''W; 1647 masl) and Yaonáhuac, Puebla (19° 56' 34''N; 97° 28' 11''W; 1272 masl). The plant material was collected during August 2017 and September 2018 and subsequently certified by MSc Ramiro Cruz-Durán. A holotype was deposited in the FCME-UNAM herbarium with voucher number 157193. The urticating hairs of *C. multilobus* were carefully removed from the plant samples with a sterile surgical blade. The endogenous fluid was squeezed (~2.5 mL in each year) from the prickles using a laboratory pincer and then, stored in sterile glass vials at -80 °C until use. The fluid was used in further chemical and antibacterial analyses.

Chemicals

Histamine, dimethylethylamine, serotonin, oxalic acid, isobutyl chloroformate, rezasurin, ampicillin, and the acetylcholine quantification kit were obtained from Sigma-Aldrich Co. 2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide (XTT dye) was purchased from ThermoFisher Scientific. Formic acid and HPLC solvents were obtained from J.T. Baker. Terbinafine was obtained from MediMart Labs®

Chemical characterization of the prickle's fluid

Histamine and dimethylethylamine were detected by the GC-MS/isobutyl chloroformate derivative protocol described by Fernandes *et al.* (2001). The analysis of these compounds was carried out in a Hewlett Packard 6890 II series coupled to an HP-5 capillary column (30 m 0.25 mm, 0.25 μ m phenyl dimethylpolysiloxane, 5:95). The detection of serotonin (5-hydroxytryptamine), oxalic acid, tartaric acid and formic acid was carried out by the RP-HPLC conditions described by Fu *et al.* (2006). Twenty microliters of the endogenous fluid were injected in a Hewlett Packard 1050 system coupled to a HP G1306A diode array detector, equipped with a Varian Chromosphere C₁₈ column (5 μ m, 250 mm \times 4.6 mm). All the assays were done in triplicate. The concentration of each compound in the fluid was calculated in μ g mL⁻¹ from calibration curves (0.2-200 μ g mL⁻¹) prepared with authentic standards (commercially obtained).

Antimicrobial assays

The phytopathogenic *Pseudomonas syringae* pv. tabaci TBR2004, *P. syringae* pv. tomato DC3000 and *Clavibacter michiganensis* AB299158 were used for antibacterial assays. These microorganisms were evaluated by the broth microdilution method using resazurin as indicator of cell viability three times in quintuplicate (n = 15) (Sarker *et al.*, 2007). The intact fluid was evaluated through dose response curves of 5-300 μ L mL⁻¹. Antifungal assays were done using the phytopathogenic fungi *Fusarium oxysporum* ATCC 62506 and *Phytophthora capsici* ATCC 15399 through dose response curves of 5-300 μ L mL⁻¹. These microorganisms were subjected to the XTT antifungal assay method (Loures *et al.*, 2015). Histamine, dimethylethylamine, acetylcholine, serotonin, oxalic acid, formic acid were individually assayed in the same bacterial and fungal pathogens (5-300 μ g mL⁻¹). Ampicillin and terbinafine were used as antimicrobials of reference.

Statistical analysis

MIC values were obtained in quintuplicate three times (n=15) and then subjected to ANOVA coupled to Tukey test ($p < 0.01$) using GraphPad Prism 7.02 software.

RESULTS

Chemical profiling

Traces of acetylcholine were detected in the fluid of *C. multilobus* collected from samples of Puebla and Oaxaca during the studies years (Table 1). However, the analytical approaches based on GC-MS revealed that the fluid contained high endogenous levels of histamine and low levels of dimethylethylamine.

Table 1. Pain-inducing toxins detected in the endogenous fluid from *C. multilobus* in the states of Puebla (P2017-P218) and Oaxaca (O2017-2018) during the years 2017-2018.

Compound	P2017	O2017	P2018	O2018
Histamine	17.3 \pm 0.55	24.3 \pm 0.15	31.5 \pm 1.1	28.4 \pm 1.3
Dimethylethylamine	2.5 \pm 0.02	3.6 \pm 0.11	2.9 \pm 0.05	1.7 \pm 0.04
Acetylcholine	1.3 \pm 0.04	2.2 \pm 0.16	0.6 \pm 0.03	2.7 \pm 0.09
Serotonin	24.6 \pm 1.1	36.3 \pm 1.4	45.8 \pm 1.6	31.4 \pm 1.9
Oxalic acid	5.7 \pm 0.13	9.6 \pm 0.17	3.1 \pm 0.19	2.2 \pm 0.08
Formic acid	0.9 \pm 0.12	2.3 \pm 0.18	1.8 \pm 0.10	4.7 \pm 0.24

The concentrations are presented in μ g mL⁻¹ plus or minus SD (n = 3).

The mass spectra of authentic standards of histamine and dimethylethylamine and those obtained from the analyzed samples were identical, confirming the presence of both amines in the natural fluid (fig. 2). Fragmentation pattern for derivatized histamine was 28 (80) 41 (43) 54 (23) 82 (100) 94 (95) 111 M^+ (10) and for dimethylethylamine was 18(15) 28(100) 32 (25) 41 (27) 56 (15) 73 M^+ (8). The levels of histamine were corroborated by using RP-HPLC. However, this technique revealed other substances with known inflammatory activity (fig. 3). Among these compounds, traces of oxalic acid and formic acid were also observed. According to our results, serotonin was the most abundant compound found in the endogenous fluid of the samples collected in the states of Oaxaca and Puebla during the studied years.

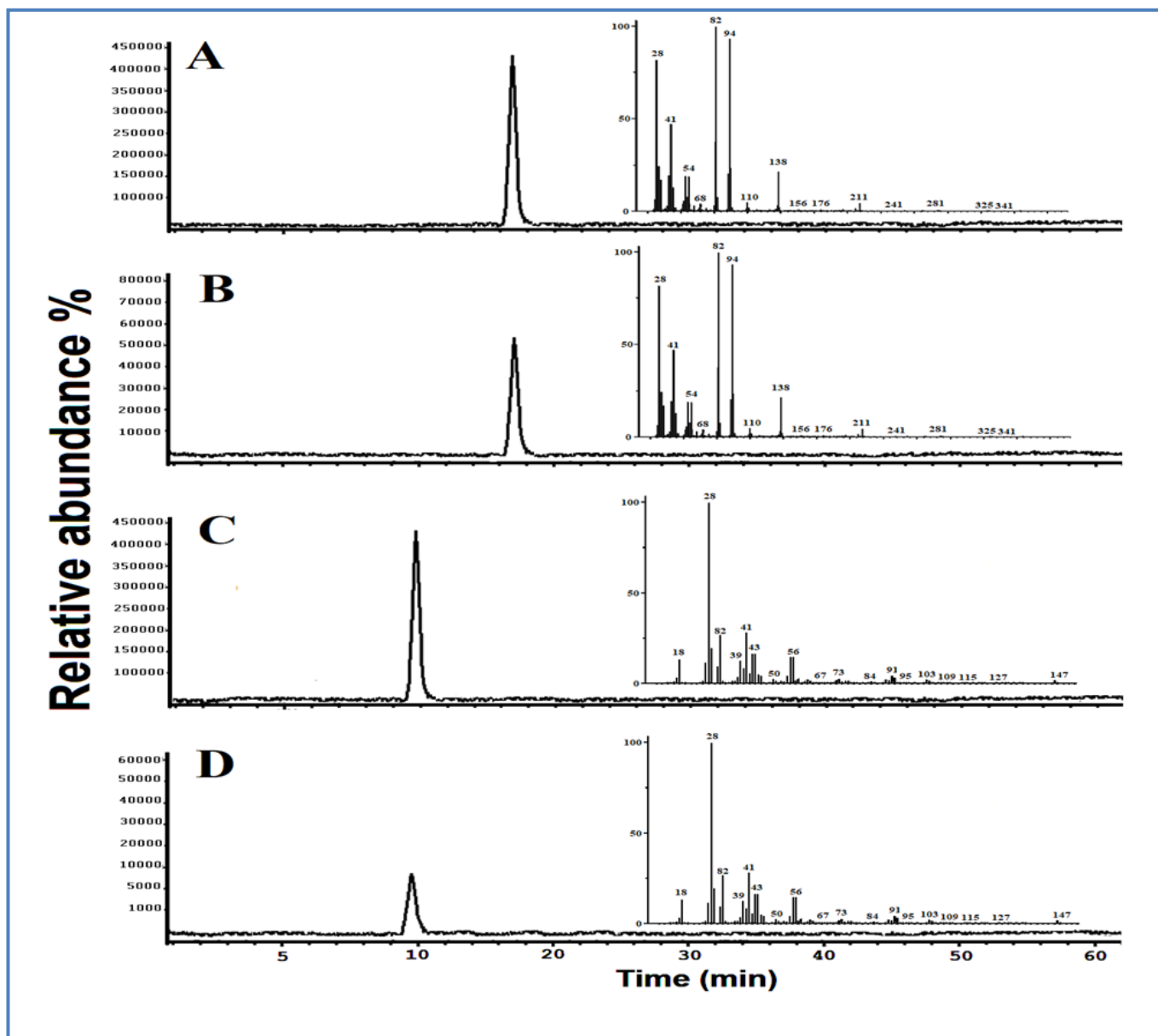


Fig. 2. TICs of GC-MS using the authentic standard of derivatized histamine (A). The presence of histamine in the derivatized fluid of *C. multilobus* is confirmed (B). The endogenous levels of dimethylethylamine (C) were also corroborated in the derivatized fluid (D). The mass spectrum (70 eV) of each compound is showed.

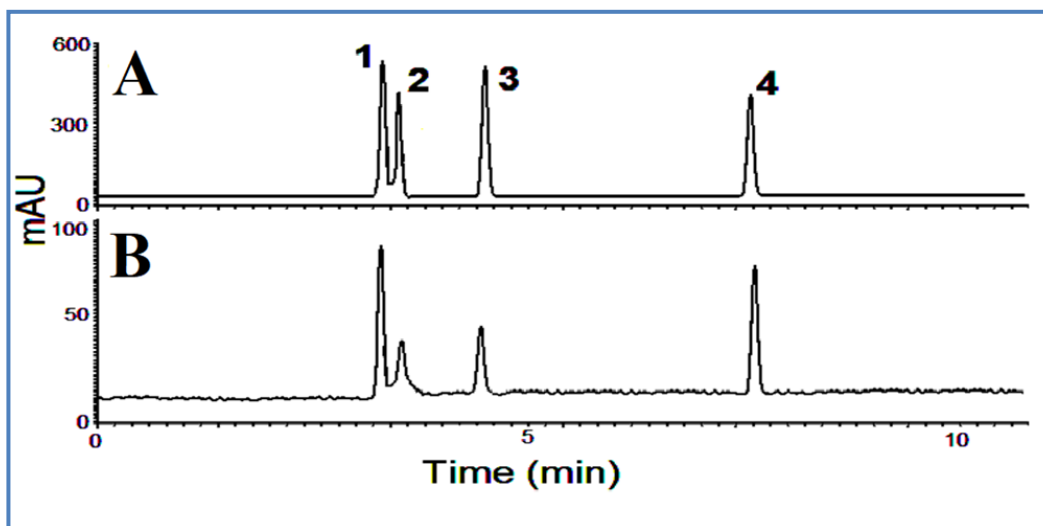


Fig. 3. A, HPLC-DAD for the simultaneous detection of authentic standards of histamine (1), oxalic acid (2), formic acid (3) and serotonin (4). B, HPLC profile of the natural fluid from the prickles of *Cnidocolus multilobus*.

The quantification of these metabolites did not reveal decreasing or increasing tendencies associated to the sites and dates of collection. At least, under the analytical conditions previously described.

Antimicrobial effects

The prickle's fluid showed inhibitory properties in the four phytopathogens assayed except for *C. michiganensis*. Remarkably, the fluid showed a potent growth inhibitory activity against the opportunistic *Fusarium oxysporum* (Table 2).

Table 2. MIC of the endogenous fluid from *C. multilobus* and its main constituents on *Pseudomonas syringae* pv. *tabaci* TBR2004 (*P.s. tabaci*), *Pseudomonas syringae* pv. *tomato* DC3000 (*P.s. tomato*), *Clavibacter michiganensis* AB299158 (*C.m.*), *Fusarium oxysporum* ATCC 62506 (*F.o.*) and *Phytophthora capsici* ATCC 15399 (*P.c.*).

Component	<i>P.s. tabaci</i>	<i>P.s. tomato</i>	<i>C.m.</i>	<i>F.o.</i>	<i>P.c.</i>
Histamine	116.2 ± 1.6 ^a	145.1 ± 2.2 ^a	-	162.3 ± 0.9 ^a	219.5 ± 0.5 ^b
Dimethylethylamine	-	-	-	210.8 ± 1.8 ^b	231.7 ± 1.3 ^b
Acetylcholine	-	-	-	-	-
Serotonin	-	-	-	-	-
Oxalic acid	-	-	-	251.7 ± 1.3 ^a	276.8 ± 0.2 ^a
Formic acid	-	-	213.6 ± 2.5	143.6 ± 1.1 ^c	185.9 ± 1.6 ^c
^Y Endogenous fluid	122.2 ± 1.3 ^a	105.8 ± 1.1 ^b	-	97.4 ± 0.8 ^d	163.2 ± 0.7 ^c
Ampicillin	3.4 ± 0.02	2.9 ± 0.03	7.3 ± 0.01		
Terbinafine				12.6 ± 0.14	8.6 ± 0.11

The MIC values for authentic standards ($\mu\text{g mL}^{-1}$) and natural fluid ($\mu\text{g mL}^{-1}$). ^Y Results expressed in $\mu\text{L mL}^{-1}$ for the fluid extracted from samples collected in Puebla, Mexico. Results are presented as the mean plus or minus standard deviation (n = 15). Means with diverse letter indicate statistically significant differences ($p < 0.01$) among tested compounds.

The individual assessment of the fluid's constituents revealed higher MIC values than those of the natural fluid. Interestingly, the evaluation of histamine revealed an inhibitory activity against two patovars of *Pseudomonas syringae* and two filamentous fungi assayed. According to the MIC obtained, histamine was particularly effective against *P. syringae* pv. tabaci whereas the natural fluid was more effective than histamine on *P. syringae* pv. tomato ($p < 0.01$). The endogenous has the best MIC value on *F. oxysporum* ($p < 0.01$). No inhibitory effect was determined for *C. michiganensis*. Oxalic acid and formic acid also produced a growth inhibitory activity against *Fusarium oxysporum* and *Phytophthora capsici* (Table 2).

DISCUSSION

Endogenous levels of histamine were previously reported in the stinging trichomes of *C. oligandrus* (Cordeior *et al.*, 1983). On the contrary, this inflammatory toxin was undetectable in the fluid of *C. texanus* (Lookadoo and Pollard *et al.*, 1991). During the studied years, histamine was the second most abundant inflammatory compound found in the endogenous fluid of the samples collected in Puebla and Oaxaca (Table 1). Remarkably, this is the first report describing the presence of dimethylethylamine as a putative pain inducing toxin of *C. multilobus*. Previous studies confirmed traces of dimethylethylamine dissolved in the oil of *Pelargonium graveolens* (Tallon *et al.*, 2012). Dimethylethylamine is known as a hazardous compound because of its acute irritant effect in eye mucosa and skin (Tallon *et al.*, 2012). Small amounts of oxalic acid and formic acid were also detected in the endogenous fluid of the stinging hairs. These organic acids and tartaric acid were considered as the principal toxins found in the fluid of prickles from *Urtica thunbergiana* (Fu *et al.*, 2006). No insights of tartaric acid were observed for *C. multilobus*.

Our findings suggests that histamine is strongly related to the acute inflammatory effects of *C. multilobus* since this compound possesses a confirmed inflammatory activity in mammal species (Cordeior *et al.*, 1983; Lookadoo and Pollard, 1991; Fu *et al.*, 2007). The unusual combination of serotonin, histamine, acetylcholine and dimethylethylamine, is reported for the first time in the endogenous fluid of the stinging hairs of a plant species (Cordeior *et al.*, 1983; Lookadoo and Pollard, 1991; Fu *et al.*, 2007). Under our analytical conditions, the endogenous levels of metabolites showed substantial fluctuations. These variations should be produced by biotic and abiotic factors associated to specific geographical locations. Due to this fact, conclusive differences in metabolite accumulation among studies samples cannot be endorsed. At least, under the analytical conditions described in this investigation.

The ecological role of the extrafloral nectar and the fluid of glandular trichomes is usually associated with attractant or repellent activities on insects and microorganisms (Kessler and Baldwin, 2007). Particularly, the function of plant stinging hairs is conferred to the mechanical and chemical defense against herbivores (Cordeior *et al.*, 1983; Lookadoo and Pollard, 1991; Fu *et al.*, 2007). Nevertheless, the effect of fluid's stinging hairs on phytopathogenic microorganisms has not been investigated until now. To the best of our knowledge, this is the first work addressing the possible effect of prickle's fluid on opportunistic phytopathogens. The results of the antimicrobial tests strongly suggest that the endogenous fluid of *C. multilobus* may be involved in the chemical defense against specific microorganisms. This evidence also suggest that the fluid's antimicrobial effect has a different intensity and modes of action on the pathogens assayed. These issues should be further addressed.

The individual assessment of single constituents revealed higher MIC values than those of the natural fluid suggesting that the antimicrobial effect is produced by the synergic activity of fluid metabolites. However, the presence of small peptides and other polar toxins dissolved in the fluid cannot be discarded. As expected, antimicrobials of reference had a better MIC values than those of the fluid and chemical constituents. Undoubtedly, the moderate activity of the prickle's fluid and its main toxins is demonstrated.

One of the most relevant findings of this investigation is the antimicrobial activity of histamine. Up to our knowledge, there is not any available report on the antimicrobial effect of this compound. It is known that the role of common amines such as putrescine, spermidine and spermine is associated with the adaptive response of microorganisms (Valdés-Santiago and Ruiz-Herrera, 2013). Nevertheless, analogue functions of histamine need to be further investigated. In addition, more investigation is required to elucidate the mode of action of histamine in specific microorganisms. The effect of organic acids on the normal growth of fungal species has been previously examined (Hassan *et al.*, 2015), showing that oxalic acid and formic acid decrease the production of mycotoxins in fungi (Hassan *et al.*, 2015). According to the experimental evidence obtained in the present investigation, only formic acid produced an inhibitory activity on *Clavibacter michiganensis*. Similar results have been attributed to changes in pH of culture medium for Gram positive bacteria (Östling and Lindgren, 1993).

CONCLUSIONS

The endogenous fluid of the stinging hairs from *C. multilobus* contained known substances with inflammatory activity such as histamine, dimethylethylamine, acetylcholine, serotonin, oxalic acid and formic acid. According with our analytical evidences, there was not a clear relationship among the accumulation of these compounds, geographical location and time of collection. However, serotonin and histamine were the most abundant compounds dissolved in the natural fluid from the samples studied. Some organic acids considered as pain-inducing compounds such as oxalic acid and formic acid were simultaneously detected. Histamine and the endogenous fluid exhibited substantial antimicrobial activity against two *Pseudomonas syringae* pathovars and two phytopathogenic fungi, suggesting their putative role as antimicrobials. However, the endogenous fluid was particularly effective against *F. oxysporum*. The individual assessment of the main constituents revealed histamine as an unnoticed antimicrobial agent.

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