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# Inhibitory effects of bark chemicals of host *Ipomoea murucoides* on seed germination of epiphyte *Tillandsia recurvata*

S. VALENCIA-DÍAZ[1,2*], A. FLORES-PALACIOS[2], V. RODRÍGUEZ-LÓPEZ[3] and A. R. JIMÉNEZ-APARICIO[1]

Centro de Investigación en Biodiversidad y Conservación (CIByC),
Universidad Autónoma del Estado de Morelos, Av. Universidad 1001, Col. Chamilpa,
C.P. 62209, Cuernavaca, Morelos, México.
E. Mail: susana.valencia@uaem.mx , susivalencia@yahoo.com.mx

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## ABSTRACT

This study determined the chemical contents of dichloromethane bark extract of *I. murucoides* that inhibited the seed germination in *T. recurvata*. Dichloromethane bark extract was subjected to two consecutive fractionations. The inhibition (%) of *T. recurvata* seed germination was determined for each resulting fractions. Using gas chromatography-mass spectrometry (GCMS), the components of primary fraction with the highest inhibition were analyzed, as well as secondary fractions derived from this primary fraction. The primary fraction G ($CH_2Cl_2$:AcOEt (v/v 1:1)) had the highest inhibitory effects on *T. recurvata* seed germination. The secondary fractions G1 (Hex:$CH_2Cl_2$ (mean concentration v/v 3:7)), G5 (CH2:Cl2:AcOEt (v/v 7:3)) and G7 (MeOH) were most inhibitory. Among the compounds of active primary fraction and secondary fractions G5 and G7, *n*-hexadecanoic acid were identified as compounds with possible allelopathic activity. However, these were not present in secondary fraction G1, it had the allelochemical 3,7,11,15-tetramethyl-2-hexadecen-1-ol. Results indicated that there were three active secondary fractions with different chemical composition but none of these inhibited the seed germination of *T. recurvata.* The inhibitory effects of dichloromethane extract of *I. murucoides* tends to diminish with fractionation, it is possible that the activity of components may be greater when they act together than when they act individually, However, further research is needed in this direction.

**Key Words:** Allelopathy, Central Mexico, epiphytic bromeliad, Fractions, GCMS, germination, *Ipomoea murucoides, Tillandsia recurvata,* tropical dry forest.

## INTRODUCTION

True epiphytes (holoepiphytes) are plants that spend their lives on other plants (hosts) without damaging them (4), however, certain hosts can interfere with epiphyte colonization by inhibiting the germination of their seeds through chemical mechanisms (23). Some hosts of genus *Ipomoea* support epiphytes but in certain environments, like tropical dry forests of central México, these hosts showed less abundance of bromeliad epiphytes than expected (24). Laboratory tests have suggested that intermediate polarity extracts from these hosts inhibits the germination in these bromeliads, possibly through the

[*]Correspondence author, [1]Instituto Politécnico Nacional (IPN). Centro de Desarrollo de Productos Bióticos (CEPROBI). Departamento de Biotecnología. Carretera Yautepec-Jojutla Km 8.5. Col. San Isidro. C. P. 62273. Yautepec, Morelos, México. [2]Current address: Centro de Investigación en Biodiversidad y Conservación (CIByC), Universidad Autónoma del Estado de Morelos, Av. Universidad 1001, Col. Chamilpa, C.P. 62209, Cuernavaca, Morelos, México. [3]Facultad de Farmacia, Universidad Autónoma del Estado de Morelos, Av. Universidad 1001, Col. Chamilpa, C.P. 62209, Cuernavaca, Morelos, México.

presence of phytol (3,7,11,15-tetramethyl-2-hexadecen-1-ol ) or palmitic acid (*n*-hexadecanoic acid) (23).

Plants of genus *Ipomoea* may have mechanisms that enable them to withstand herbivores or competitors; for example, *I. hederaceae* (L.) Jacquin resists the attack of herbivorous insects through constitutive defences (trichomes) (22). Moreover, it is known that the *Ipomoea* genus produces allelopathically active alkaloids and resin glycosides (3,10,15). *Ipomoea tricolor* an allelopathic plant reduces the seed germination and seedling growth through tricolorin A, a phytotoxic compound of resin glycosides (3). Species of genus *Ipomoea* (like *I. tricolor*, *I. pauciflora* and *I. murucoides*) contain chemical compounds that interact with other plant species. *I. murucoides* inhibits the germination of epiphyte *Tillandsia recurvata* through dichloromethane bark extract.

The aqueous and organic bark extracts of *I. murucoides* and *I. pauciflora*, inhibited the seed germination (58%) of *T. recurvata*, through a dichloromethane extract (23), although the components responsible for such inhibition have not yet been identified. This study aimed to determine the chemical contents of dichloromethane bark extract of *I. murucoides* that inhibits the seed germination in *T. recurvata*. It was hypothesized that allelopathically active fractions would share some chemical compounds and they would be of intermediate polarity, because the allelopathic compounds (tricolorin A) and active extracts of this genus have been obtained by solvents of intermediate polarity [chloroform (polarity index 4.1) and dichloromethane (polarity index 3.1)] (15,23).

## MATERIALS AND METHODS

### I. Study species and collection site

*Ipomoea murucoides* (Convolvulaceae) is a deciduous tree (8 m in height) and is common in low deciduous tropical forests of central and southern Mexico, and flowers from October to March (5). *Tillandsia recurvata* is an atmospheric bromeliad, i.e. it obtains its nutrients from the air. It is widely distributed (7) and is considered dominant in some epiphyte communities (8,24) like in Tropical dry forest at San Andrés de la Cal, Tepoztlán, Morelos (18º57'22.2'' W, 99º06'50.2'' N, 1495 m a.s.l.) (mean annual precipitation : 1098 mm, temperature : 12 ºC and 18 ºC), Mexico (18). From this place, *Tillandsia recurvata* seeds and bark of *I. murucoides* were collected in 2008 and 2007, respectively.

### II. *I. murucoides* dichloromethane extract fractionation

The dichloromethane bark extract is most inhibitory to seed germination of *T. recurvata* (23), hence, a bioassay was conducted with fractionation from this extract. Dichloromethane extract was previously obtained from branches of *I. murcoides* (23). Bark was peeled from the branches and oven-dried (FD 115-UL, Binder) at 30°C until a constant dry weight was reached. Dry bark was ground to < 3 mm in an electric mill (PULVEX S. A. de C.V. model Mini-100). Using the 700 g dry bark powder, we did consecutive extraction by maceration at room temperature, with hexane and after using dichloromethane. After filtration, extracts were concentrated under vacuum (Rota-evaporator Buchi R-200) at 39ºC (23).

From 10 g of dichloromethane extract, we conducted two consecutive fractionations. The bio-directed fractionation consisted of assessing the effect of each fraction on the seed germination of *T. recurvata*. In this way, only the primary fraction that produced the highest inhibition was itself fractionated. Both the first and second fractionations were done with open column chromatography, using silica gel (Merck®, No. 60) in a 1:20 proportion as a stationary phase. Elution was done with different solvents and binary combinations in different proportions for each fractionation. In the same way that dichloromethane extract, fractions were concentrated under vaccum (Rota-evaporator Buchi R-200). Fractions that contained hexane and/or dichloromethane were concentrated at 39° C and those with ethyl acetate and/or methanol at 45° C.

Based on polarities of hexane (hex, 0), dichloromethane ($CH_2Cl_2$, 3.10), ethyl acetate (AcOEt, 4.4) and methanol (MeOH, 5.1) (19), we calculated the polarities for primary and secondary fractions. Polarities of binary combinations were calculated as under:

$$(Pa * Ca) + (Pb * Cb),$$

Where, $P$ : Polarity index of solvent $a$ in binary combination, $C$ : Concentration; $P$ : Polarity index of solvent $b$ in mixture.

Yield (%) was calculated as under:

$$\frac{WF \times 100}{TW}$$

Where, TW : Total weight of extract or active fraction G, WAF : Weight of fractions.

The first fractionation produced 11 fractions identified as A (Hex), B (Hex:$CH_2Cl_2$, v/v 9:1), C (Hex:$CH_2Cl_2$, v/v 7.5:2.5), D (Hex:$CH_2Cl_2$, v/v 1:1), E (Hex:$CH_2Cl_2$, v/v 2.5:7.5), F ($CH_2Cl_2$), G ($CH_2Cl_2$:AcOEt, v/v 7.5:2.5), H ($CH_2Cl_2$:AcOEt, v/v 1:1), I ($CH_2Cl_2$:AcOEt. v/v 2.5:7.5), J (AcOEt) and K(MeOH). From 1 g of primary fraction (fraction G), that caused maximum inhibition in seed germination of *T. recurvata* a second fractionation was done, which produced further fractions, three of them (Hex:$CH_2Cl_2$, v/v 4:6; Hex:$CH_2Cl_2$, v/v 3:7 and Hex:$CH_2Cl_2$, v/v 2:8) were identified as G1 because they had similar chemical content according to thin layer chromatography, G2 (Hex:$CH_2Cl_2$, v/v 1:9), G3 ($CH_2Cl_2$), two G4 ($CH_2Cl_2$:AcOEt, v/v 9:1 and $CH_2Cl_2$:AcOEt, v/v 8:2) because thin layer chromatography established similarities in the compound contents of these fractions, G5 ($CH_2Cl_2$:AcOEt, v/v 7:3), G6 ($CH_2Cl_2$:AcOEt, v/v 6:4) and G7 (MeOH). As consequence, these were reduced to 7 fractions.

**III. Germination tests**

Germination experiments were done with *T. recurvata* seeds, which were treated with the primary and secondary fractions. The concentration of fraction used in the experiments with primary and secondary fractions was equal to the most inhibitory for the dichloromethane extract (1 µg/ml of distilled water (23)). Twenty seeds were placed in each petri dish with filter paper (Whatman No. 2) as substrate. Each of 11 primary fractions plus a control consisted of five petri dishes. While each of 7-secondary fractions

plus a control consisted of four petri dishes. Three ml solution of each fraction was added to each petri dish with seeds. The control consisted of 3 ml distilled water without extract. Dimethyl sulfoxide (DMSO) at maximum concentration of 1% (v/v) was added to those fractions that were not soluble in water. Previous experiments (23) showed that DMSO has no effect on seed germination of *T. recurvata* than distilled water treatment ($t_{22}$ = -1.6, P = 0.1, mean ± 1 SE, 87.9% ± 2.2% germination in water vs. 83.7% ± 1.2% germination in DMSO). Petri dishes with seeds were placed in a bioclimatic chamber (Scorpion Scientific A 50624, México) [photoperiod:12 h light/12 h dark at 30º C] for 10 days to germinate. Seeds with no rupture of testa after 10 days were considered ungerminated (Fig. 1a) but seeds with ruptured testa were considered germinated (9) or if they were filled with water and green color (Fig. 1b).


Figure 1. (a) Ungerminated seed and (b) Germinated seeds of *T. recurvata*. Notice that germinated seeds become filled with water and greenish color.

All statistical analysis was done using the program STATISTICA (Stat Soft, Tulsa, OK, USA). As the data did not comply with the assumptions of homoscedasticity, hence, a Kruskal-Wallis analysis of variance was used (26), where treatment was fraction, including the control, and response variable was the inhibition (%) of seed germination, calculated by

$$\frac{\overline{X_0} - Xi}{n} \times 100 \ ,$$

Where, $\overline{X_0}$: Mean number of germinated seeds in control, *Xi* : Number of germinated seeds at a specific concentration (*i*) and *n* : Number of seeds (20) in an experimental unit. In this equation, a +ve value : Inhibition, a -ve value : Promotion of germination and zero : Complete absence of host bark effect. Differences between the treatments were evaluated by Duncan multiple comparison test between the medians (21).

**IV. Chemical content of fractions**

Once the most inhibitory primary fraction was identified, it was analyzed by thin layer chromatography, using aluminum plates covered in silica (60 F254, Merck®) and

benzene-ethyl acetate (8.5:1.5, v/v) as an eluent. Ceric sulfate was used as a visualizing agent the plate was also visualized using UV light at wavelengths of 254 nm and 366 nm thereafter the plate was heated one minute at 110°C. Using gas chromatography-mass spectrometry (GCMS), the chemical components of the primary and secondary fractions that produced maximum inhibition in germination were analyzed.

To determine the similarities or differences in chemical contents of secondary fractions, a cluster analysis was done (1). The variables were the secondary fractions that were grouped as per the abundance of chemical compounds detected by the GC-MS (Table 1) in each fraction. The cluster considered euclidean distances as the principal matrix and clustering was conducted with the simple linkage amalgamation, or nearest neighbor, rule. All analyses were done using the program STATISTICA (Stat Soft, Tulsa, OK, USA).

## RESULTS AND DISCUSSION

### I. Inhibitory effects of secondary fractions on seed germination in *T. recurvata*

Of the 11 fractions obtained in primary fractionation, fraction K was largest, with a yield of 3.90 g, followed by fractions A and G both with 1.11g. Yield of other fractions were: C > J > I > H > F = B >D > E (Table 2). From the secondary fractionation from fraction G, the largest fraction was G1 with a yield of 0.79 g followed by G6 (0.04 g) = G2 (0.04 g) > G7 (0.01 g) > G4 (0.006 g) = G5 (0.006 g) > G4 (0.005 g) > G3 (0.002 g).

Table 1. Chemical compounds identified by GCMS (gas chromatography mass-spectrometry) of the secondary fractions (G1-G7)

| Chemical compound | Secondary fractions | | | | | | | Molecular |
|---|---|---|---|---|---|---|---|---|
| | G1 | G2 | G3 | G4 | G5 | G6 | G7 | Formula |
| 3,7,11,15-Tetramethyl-2-hexadecen-1-ol | X | X | | | | | | $C_{20}H_{40}O$** |
| 2-Pentadecane,6,10,14-trimethyl- | X | X | X | X | X | X | X | $C_{18}H_{38}$* |
| 1-Heptadecanol | X | | | | | | | $C_{17}H_{36}O$** |
| 9,12.Octadecadienoic (*Z*,*Z*)-acid | X | | | | | | X | $C_{18}H_{32}O_2$* |
| Hexanedioic, bis(2-ethylhexyl) ester acid | X | | | | | | | $C_{22}H_4O_4$* |
| *Z*-11(13,14-Epox)tetradecen-1-ol acetate | | | | X | X | | X | $C_{13}H_{13}NO_3$** |
| *n*-hexadecanoic acid | | | | X | X | | X | $C_{16}H_{32}O_2$** |
| Isopropyl palmitate | | | | X | | | | $C_{19}H_{38}O_2$** |
| 1-decanol, 2-hexyl- | | | | X | | | X | $C_{16}H_{34}O$** |
| Octadecanoic acid | | | | X | | | | $C_{18}H_{36}O_2$** |
| 1-Octadecene | | | | | X | X | | $C_{18}H_{36}$** |
| 2-Propenoic,3-(4-methoxyphenyl)-, 2-ethylhexyl ester acid | | | | | X | | X | $C_{18}H_{26}O_3$* |
| 9,19-Cyclolanostan-3-ol, 24-methylene-, (3.beta.)- | | | | | X | | | $C_{31}H_{52}O$** |
| 1,2-Benzenedicarboxylic, bis(2-methylpropyl) ester acid | | | | | | | X | $C_{16}H_{22}O_4$* |
| Oleic acid | | | | | | | X | $C_{18}H_{34}O_2$** |
| Dotriacontane | | | | | | | X | $C_{32}H_{66}$** |

Molecular formulas were searched in web data bases:* webbook.nist.gov (14) and **www. chemspider.com (6).

For primary fractions, fraction A (hexane) had the lower polarity equal to 0, while fraction K (MeOH) had the higher polarity (5.1) (Table 2). Polarities of secondary fractions were: G1 with a mean polarity of 2.17, G2 = 2.79, G3 = 3.1, mean polarity of G4 was 3.29, G5 = 3.49, G6 = 3.62 and G7 = 5.1.

Table 2. Primary fraction polarities and yields obtained from 10 g dichloromethane extract of *Ipomoea murucoides* bark

| Fraction | Polarity | Yield (g) |
|---|---|---|
| A | 0 | 1.11 |
| B | 0.31 | 0.20 |
| C | 0.77 | 0.42 |
| D | 1.70 | 0.19 |
| E | 2.32 | 0.14 |
| F | 3.10 | 0.20 |
| G | 3.50 | 1.11 |
| H | 3.75 | 0.23 |
| I | 4.07 | 0.27 |
| J | 4.40 | 0.36 |
| K | 5.10 | 3.90 |

In germination experiment using the primary fractions, significant differences were found between treatments ($H_{11, N=60}$=34.90, P < 0.003). Fraction G caused maximum inhibition in seed germination of *T. recurvata* (Figure 2a). Significant differences were also found between treatments ($H_{7, N=32}$=17.73, P = 0.01) in germination experiment with the secondary fractions of fraction G; however, these differences were relative to the control (Fig. 2b). Fractions G1 (28.33 ± 1.66% inhibition), G5 (31.66% ± 1.66% inhibition) and G7 (33.33% ± 7.68% inhibition) produced maximum inhibition in seed germination than control, but were similar to other treatments (Figure 2b).

The components with allelopathic potential are present in certain species of genus *Ipomoea*, such as *I. tricolor*, which reduces the growth of plants in its proximity (2, 3). The organic and aqueous bark extracts of *I. pauciflora* and *I. murucoides* reduces the seed germination of *T. recurvata* (23), but *I. murucoides* was most inhibitory due to its dichloromethane extract. However, the primary fractions of dichloromethane extract of *I. murucoides* bark had similar inhibitory effects on the germination of *T. recurvata* seeds and the effect of some primary fractions was similar to control. Although fraction F had the same polarity as the dichloromethane extract, but it did not exert similar inhibitory effect on seed germination, probably because the dichloromethane extract had a wider spectrum of chemical components than fraction F and these components probably act together exerting an inhibitory effect. On the other hand fraction G was the only fraction to stand out in terms of its inhibitory activity on seed germination of *T. recurvata*. However, it did not have greater effect than dichloromethane extract. Results suggested that the chemical content involved in germination inhibition from G was specific to fraction G polarity and that the inhibitory activity diminish with fractionation.

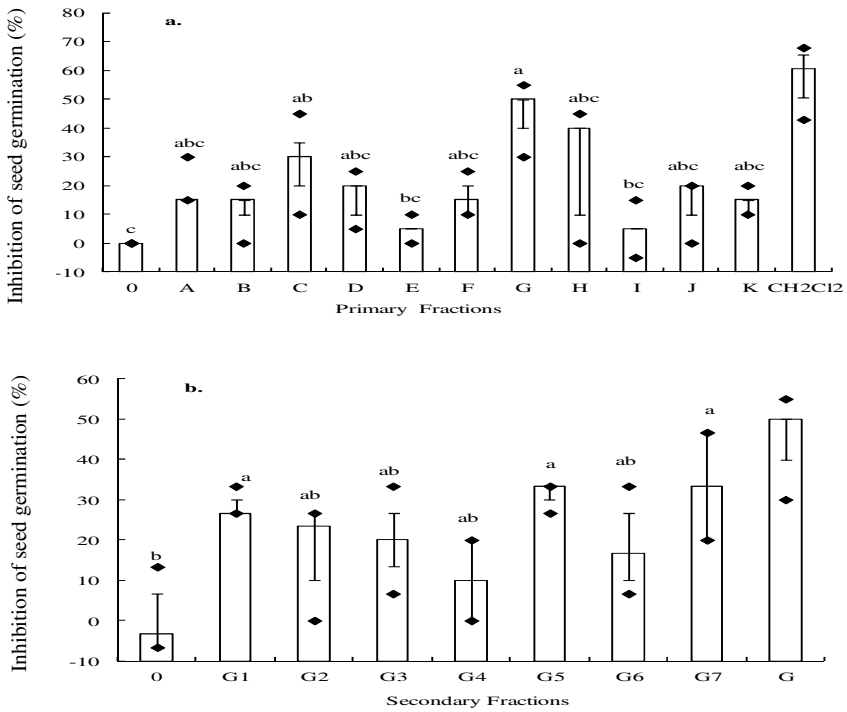


Figure 2. (a) The effect of primary fractions and of dichloromethane extract of *I. murucoides* on seed germination in *T. recurvata*. The dichloromethane extract exerted higher inhibition, (b) Effect of secondary fractions and primary fraction G, on seed germination in *T. recurvata*. Effects of secondary fraction are similar to fraction G. Each column total length represents the median, dispersion lines are the 25 % and 75% quartiles and solid rhombus indicate minimum and maximum values. Different letters indicate significant differences ($P < 0.05$) between medians.

Unlike the studies conducted with *I. tricolor*, where fractionation of chloroformic extract indicated the presence of a single compound (tricolorin A) that inhibits the root growth in other species [*Amaranthus leucocarpus* Watts. and *Echinochloa crus-galli* (L.) Beauv. (15)], it seemed that fraction G was less inhibitory than dichloromethane extract (57.91% ± 5.40%). However, there were no significant differences between the dichloromethane extract and G fraction.

The secondary fractions contained shared content components (Fig. 3, Table 1), which made them similar to each other, but fraction G7 had the maximum number of components that were different than other fractions (Fig. 3, Table 1).

The reduction in seed germination of *T. recurvata* by the secondary fractions tends to diminish and the effect was not exclusive to one fraction, but was produced instead by 3-different fractions. It is worth noting that the main differences occurred between the secondary fractions and the control. This is probably because the components of certain mixtures have a synergistic effect when they act in unison. For example, the

mixtures of phenolic acids reduces the seed germination inhibition in *Plantago lanceolata* L., but the opposite occurs when these compounds act separately (17). Likewise, while the methanolic extract of stem of *Pterodon emarginatus* Vogel inhibits the germination of grass *Panicum máximum* Jacq., fractions of this extract do not show the same inhibitory activity (12). Another possible explanation for the reduced activity of fractions relative to extract is that the activity of these components may diminish with time, but this need to be probed.

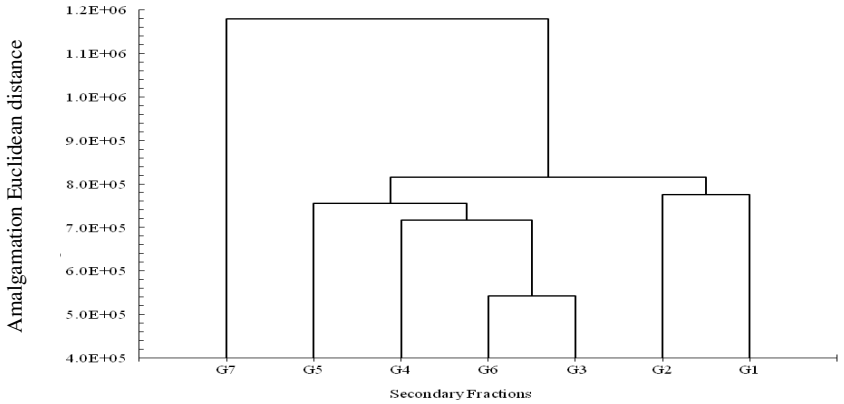


Figure 3. Dendrogram of secondary fractions. Distances indicate the degree of difference in chemical content between fractions.

**II. Fractions chemical content**

The chromatographic plate of fraction G separated into nine components, which were mainly distributed on the high part of the plate (Rf values > 0.5), signify low polarity in these components. The GC-MS results indicated that the fraction G contains 19 chemical compounds, but those with higher abundances were 3,7,11,15-tetramethyl-2-hexadecen-1-ol (phytol), cyclopropanoic acid, *n*-hexadecanoic acid (palmitic acid) and isopropyl palmitate.

In common with the dichloromethane extract (23), the 3,7,11,15-Tetramethyl-2-hexadecen-1-ol and *n*-hexadecanoic acid were also found in fraction G. Both of these compounds could act as growth inhibitors (13,16). According to the cluster analysis, the secondary fractions G1, G5 and G7 had the highest inhibitory effects but presented differences in their components. However, the fraction G7 was much different, since it was separated from other fractions. In common with primary fraction G, secondary fractions G5 and G7 also contained *n*-hexadecanoic acid among their components; however, the presence of this compound cannot be related alone to the inhibition of seed germination in *T. recurvata*, because fraction G5 had 1-Octadecene which promotes the growth of photosynthetic bacteria (25). Fraction G7, on the other hand, contained 1,2-benzenedicarboxylic, bis(2-methylpropyl) ester acid which was found in exudates of hot pepper and act as an inhibitor (10). Fraction G1, the most inhibitory of the secondary fractions, did not contain palmitic acid but it contains the diterpene phytol, which constitute part of chlorophyll molecule and it can also act as root growth inhibitor (13).

In this study, we did not find one single fraction that stood out as an inhibitor of *T. recurvata* seed germination, but instead we found three fractions that differed in composition, acting as possible allelochemicals: palmitic acid, phytol, 1-octadecene and 1,2- benzenedicarboxylic, bis(2-methylpropyl) ester acid, which according to literature have allelopathic potential. On the other hand, inhibitory activity diminished in the secondary fractionation. It is possible that the components of extract exert a greater effect in unison than individually, but it needs to be probed in future studies.

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