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Evaluation of antimicrobial and antifungal activity of hydroethanolic extracts of *in vitro* and *ex vitro* seedlings of *Dionaea muscipula* J. Ellis

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ABSTRACT

Objective: To evaluate the antibacterial and antifungal activity of hydroethanolic extracts of *Dionaea muscipula* J. Ellis against bacteria models and fungi of agricultural importance.

Design/Methodology/Approach: *In vitro* plants of *D. muscipula* were propagated and acclimatized for three months. The antibacterial activity of the hydroethanolic extracts against *Escherichia coli* and *Bacillus cereus* was evaluated, and the antifungal activity against *Aspergillus niger*, *Fusarium oxysporum* and *Pseudocercospora fijiensis*. Analysis of variance (ANOVA) was carried out to compare the means obtained with Tukey's test ($p \leq 0.05$).

Results: The hydroethanolic extracts of *in vitro* and acclimatized *D. muscipula* presented bacteriostatic activity against the bacteria *E. coli* (65.20 and 69.78% of inhibition) and *B. cereus* (91.75 and 92.61% of inhibition), and antifungal activity against the fungus *P. fijiensis* of 7.56 and 14.21% of inhibition, respectively.

Study Limitations/Implications: The hydroethanolic extracts of *in vitro* and acclimatized *D. muscipula* did not show antifungal activity against *A. niger* and *F. oxysporum*.

Findings/Conclusions: The hydroethanolic extracts of *in vitro* and acclimatized *D. muscipula* presented bacteriostatic activity against bacteria *E. coli* and *B. cereus* and antifungal activity against the fungus *P. fijiensis*.

Keywords: antibacterial activity, antifungal activity, *Dionaea muscipula*, carnivore plants.

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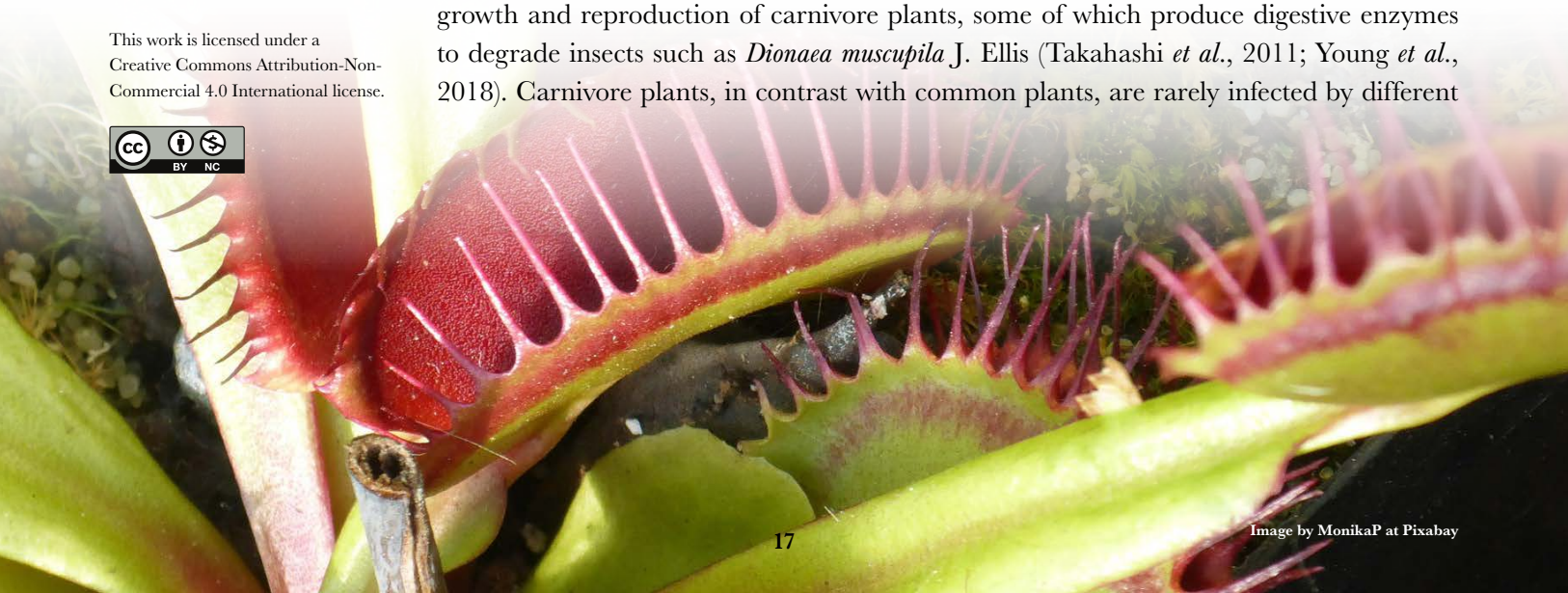
INTRODUCTION

Carnivore plants have attracted the general public due to their exotic nature and the ability to attract, capture and digest insects (Ellison and Adamec, 2018); carnivorism provides nitrogen (N), phosphorus (P) and other supplementary mineral nutrients, improving the growth and reproduction of carnivore plants, some of which produce digestive enzymes to degrade insects such as *Dionaea muscupila* J. Ellis (Takahashi *et al.*, 2011; Young *et al.*, 2018). Carnivore plants, in contrast with common plants, are rarely infected by different



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pathogenic agents carried by insects, such as bacteria, viruses and fungi, which can suggest the presence of defense mechanisms or production of secondary metabolites in the plant (Ogihara *et al.*, 2013). It has been reported that the extracts from the tissues of *D. muscipula* present antioxidant (Makowski *et al.*, 2020), anticancer (Kawiak *et al.*, 2019), and antibacterial properties against bacteria such as *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* (Królicka *et al.*, 2008). However, despite being of utmost importance, the fungicide activity against phytopathogenic fungi has been scarcely studied. Therefore, the antibacterial and antifungal activity of hydroethanolic extracts of *in vitro* and acclimatized *D. muscipula* against model pathogens and of agricultural importance (*E. coli*, *B. cereus*, *A. niger*, *F. oxysporum* and *P. fijiensis*) was evaluated.

MATERIALS AND METHODS

D. muscipula plants were used, belonging to the active collection of the Biotechnology and Plant Cryobiology laboratory from the Chemistry School at Universidad Veracruzana, from *in vitro* culture in a semisolid MS medium (Murashige & Skoog, 1962) at 1/3 of concentration, supplemented with 3% sucrose (Fermont®) and 2.5 g L^{-1} phytagel (Sigma-Aldrich®) as jellifying agent. The pH was adjusted to 5.8±0.1, with HCl 0.1 N (Fermont®) and NaOH 0.1 N (Fermont®) and sterilized in autoclave at 120 °C and 115 kPa for 15 min. Later, *D. muscipula* seedlings were sub-cultured in containers with 30 mL of culture medium in a laminar flow hood. The explants were incubated at 25±1 °C with irradiation of 40 μmolm^{-2} s^{-1} provided by fluorescent lamps of 60 W (Osram®) with a photoperiod of 16 hours light and 8 hours darkness. *In vitro* plants of *D. muscipula* were acclimatized during three months using Peat Moss and agrolite (1:1) as substrate, in controlled greenhouse conditions under radiation with natural light of 130 μmol m^{-2} s^{-1}, 30±2 °C and 60±5% of HR. In addition, a sample of the specimen was deposited in the CORU Herbarium Dr. Jerzy Rzedowski Rotter of the Biological and Agricultural Sciences School at Universidad Veracruzana, Córdoba, Veracruz, with number of identification certificates SVH001.

Preparation of hydroethanolic extracts

Fifteen (15) g of *in vitro* and acclimatized *D. muscipula* plants were used, three months old, in sterile conditions, washed with distilled water and dried with filter paper, frozen with liquid nitrogen and pulverized in a mortar with pestle; then, they were macerated by adding 100 mL of a 80:20 ethanol:water (% v/v) solution. The extracts were poured into sterile Erlenmeyer flasks covered with aluminum paper to avoid photo oxidation, the hydroethanolic extracts were concentrated in rotary evaporator (IKA®) to a tenth of the original volume, frozen to −6±2 °C and liquid nitrogen was added to lyophilize at −86 °C and 0.01 mbar in a freeze-drier (LABCONCO® 7.2).

Biological material (bacteria and fungi)

Bacteria cultures (*E. coli* and *B. cereus*) and fungal cultures (*A. niger*, *F. oxysporum*, and *P. fijiensis*) were used, which belonged to the active collection of the Biotechnology and Food Analysis laboratory of the Chemistry School at Universidad Veracruzana. To cultivate *E. coli*, 22.5 g of trypticase soy broth (CSoT) were used and dissolved in 750 mL water, taken

to boiling point in constant agitation; the pH was adjusted to 7.3±0.2. Then, 50 mL were poured in Erlenmeyer flasks of 125 mL and sterilized in autoclave at 120 °C and 115 kPa for 15 min. Nutritional broth was used to cultivate *B. cereus*, 6 g were weighed and diluted in 750 mL of water, it was heated and agitated until its complete dilution. The pH was adjusted to 6.9±0.2, and 50 mL were poured in Erlenmeyer flasks of 125 mL and sterilized in autoclave at 120 °C and 115 kPa for 15 min.

To cultivate *A. niger*, *F. oxysporum*, and *P. fijiensis*, 7.8 g of potato dextrose agar (PDA) (MCDLab) were used, 200 mL of distilled water were added, and this was left to rest for 15 min; then, it was heated for 1 min in constant agitation until its dilution, sterilized in autoclave at 120 °C and 115 kPa for 15 min, and left to cool until 45 °C. In a laminar flow hood, the PDA medium was poured into sterile Petri dishes and left to solidify for 15 min. Finally, for the inoculation, squares of 1 cm^2 of agar from a Petri dish with the fungus in active growth were used and incubated at 28 °C.

Evaluation of the antibacterial activity

The hydroethanolic extracts were re-suspended with sterile distilled water to obtain a concentration of 1% and filtered using a membrane (Millex®), with a pore size of 0.22 μm to eliminate residues.

The evaluation of the antibacterial activity of hydroethanolic extracts was carried out through the poisoned medium method described by Ochoa Fuentes *et al.* (2012). The strains of *E. coli* and *B. cereus* were activated in 125 mL flasks, adding 50 μL of the inoculum from each bacterium in 50 mL of CsoT medium and nutritional broth, respectively. Later, after 18 hours of incubation, 45 mL of fresh medium were taken and inoculated with 5 mL of culture medium activated with *E. coli* and *B. cereus*.

For the evaluation of the antibacterial activity, 150 μL of the extracts at a concentration of 1% were added to the culture medium inoculated with *E. coli* and *B. cereus*, 150 μL of broad-spectrum commercial antibiotic (Ceftriaxone) were used as positive control, the negative control consisted in the inoculated culture medium with the respective bacteria, and as target the culture mediums without inoculation were used added with 150 μL of the hydroethanolic extract.

A bacterial growth kinetic of 12 h was conducted. Each hour an aliquot of 1000 μL was taken in a laminar flow hood to avoid crossed contamination and the absorbance was measured at 620 nm in spectrophotometer (JENWAY 6305™). Each treatment was carried out by triplicate.

Evaluation of the antifungal activity

The evaluation of antifungal activity of the extracts of *D. muscipula* was determined through the "empty box" method described by Cruz-Cruz *et al.* (2010). Initially, Petri dishes were filled to ¼ of its volume with "agar-agar" medium (Omnichem®) and it was left to solidify, plastic cylinder molds (0.5 cm of diameter) were placed to form a "well" in the central part of the Petri dish. In parallel, 10 inoculates of 1 cm^2 from each fungus were mixed with 400 mL of PDA medium at 36 °C and the resulting suspensions were served in Petri dishes that contained the solidified "agar-agar" medium with the plastic cylinder

mold. For all the evaluations, 150 μL of the hydroethanolic extracts at 1% were used to fill the wells, and for the negative control 150 μL of a 80:20 ethanol:water (% v/v) mixture were added to the wells, and for positive control 15 μL of Neomicol®.

Later, the Petri dishes that contained *A. niger* and *F. oxysporum* were incubated during 7 days at 28 °C, and for *P. fijiensis* 15 days at 28 °C. Each treatment was carried out by triplicate. The evaluation of the antifungal activity was conducted by measuring the inhibition area through the ImageJ version 2018 software.

Experimental design and statistical analysis

A completely random experimental design was used. All the assays were performed by triplicate. The data were processed using the MINITAB 18 STATISTICAL software and the statistical analysis was carried out through an analysis of variance (ANOVA) and a means comparison using Tukey's test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Evaluation of the antibacterial activity of hydroethanolic extracts of *in vitro* and acclimatized *D. muscipula* against *E. coli* and *B. cereus*

The hydroethanolic extracts of *in vitro* and acclimatized *D. muscipula* presented bacteriostatic activity against *E. coli* (Table 1), showing inhibition of microbial growth of 65.20±0.52 and 69.78±0.72%, respectively. The positive controls (Ceftriaxone), inhibited 97.90±0.83 and 96.25±0.63% of microbial growth of *E. coli* in both evaluations, showing significant difference compared to the treatment with hydroethanolic extracts. The negative controls (inoculated medium) did not present inhibition, while in the blanks (cultivation medium) bacterial growth was not observed.

Authors like Tafur *et al.* (2011) report that gram-negative bacteria have higher resistance to the secondary metabolites, which could be the reason of bacteriostatic activity. Chávez Pérez *et al.* (2017) reported that plumbagin from the tissue of *D. muscipula* provides the antibacterial properties while Tokunaga *et al.* (2004) describe that they provide protection against predators and parasites.

The results from the hydroethanolic extract of *Dionaea* against *E. coli* presented a bacteriostatic activity; that is, the extract did not completely inhibit the bacteria, but rather made it age, stopping its growth and reproduction. Makowski *et al.* (2020) reported that the elicitation with *C. sakazaki* lysates increased the bactericide properties of the *D. muscipula*

Table 1. Percentage of bacterial inhibition of hydroethanolic extracts from *in vitro* and acclimatized plantlets of *D. muscipula*.

	E. coli		***B. cereus***	
	***In vitro* plantlets (%)**	**Acclimatized plantlets (%)**	***In vitro* plantlets (%)**	**Acclimatized plantlets (%)**
Control + (Ceftriaxone)	97.90±0.83^{a}	96.25±0.63^{a}	97.57±2.71^{a}	99.20±0.57^{a}
Control − (inoculated medium)	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}	0.00±0.00^{c}
Hydroethanolic extract 1%	65.20±0.52^{b}	69.78±0.72^{b}	91.75±0.12^{b}	92.61±0.35^{b}

tissue against *E. coli*, thus suggesting that gram-negative bacteria are less sensitive to the compounds derived from *D. muscipula* tissue.

The evaluation of hydroethanolic extracts of *in vitro* and acclimatized *D. muscipula* also presented bacteriostatic activity against *B.cereus* (Table 1), showing inhibition of microbial growth of 91.75±0.12 and 92.61±0.35%, respectively. The positive controls (Ceftriaxone) inhibited 97.57±2.71 and 99.20±0.57% of microbial growth of *B. cereus* in both evaluations, showing significant difference compared to the treatment with hydroethanolic extracts. Likewise, the negative controls (inoculated medium) did not show inhibition, while in the blanks (culture medium) bacterial growth was not observed.

The bactericide activity presented by the extracts of *D. muscipula* against *B. cereus* inhibited 91.75 and 92.61% of microbial growth, being higher than those observed with *E. coli* of 65.20 and 69.78%. Manandhar *et al.* (2019) describe that the difference in the nature of the cell wall makes gram-positive bacteria more susceptible to different compounds than gram-negative bacteria. Authors like López *et al.* (2011) report that metabolites found in the extracts with antimicrobial activity are flavonoids and naphthoquinones, which owe their antibacterial activity to sharing analogue structures with antibiotics.

Evaluation of the antifungal activity of hydroethanolic extracts of *D. muscipula* against *A. niger*, *F. oxysporum* and *P. fijiensis*

The hydroethanolic extracts of *in vitro* and acclimatized *D. muscipula* did not show inhibition against phytopathogenic fungi *A. niger* and *F. oxysporum* (Table 2). This could be because the extracts of *D. muscipula* did not have the specific secondary metabolites to combat the growth of *A. niger* and *F. oxysporum*. However, the hydroethanolic extracts of *in vitro* and acclimatized *D. muscipula* presented antifungal activity against *P. fijiensis*, respectively.

The *in vitro* seedling extract showed fungal inhibition of 7.56±1.1 and the *ex vitro* seedling extract of 14.21±0.85%, with significant statistical differences. The fungal inhibition of *ex vitro* seedling extract (14.21±0.85%) was higher (0.28%) than what was observed in the positive control (Neomicol®) (13.9±1.3%), without significant statistical differences (Table 2, Figure 1).

Castillo *et al.* (2012) describe that the plants synthesize secondary metabolites with antifungal activity to stop or inhibit the development of mycelia growth or reducing the

Table 2. Percentage of fungal inhibition of hydroethanolic extracts from *in vitro* and acclimatized plantlets of *D. muscipula*.

	A. niger	***F. oxysporum***	***P. fijiensis***
Control + (Neomycol®)	11.54 ± 0.62^{a}	16.37 ± 1.17^{a}	13.93 ± 1.39^{a}
Control − (ethanol:water, 80:20)	0 ± 0^{b}	0.61 ± 0.54^{b}	0.61 ± 0.54^{c}
Hydroethanolic extract of *in vitro D. muscipula* 1%	0.36 ± 0.31^{b}	0.78 ± 0.29^{b}	7.56 ± 1.1^{b}
Hydroethanolic extract of acclimatized *D. muscipula* 1%	0.5 ± 0.14^{b}	0.61 ± 0.13^{b}	14.21 ± 0.85^{a}

Values represent the mean ± SD (standard deviation). Means with different letters in a column are statistically different (Tukey's test; $p\leq0.05$). At 15 days post-inoculation.

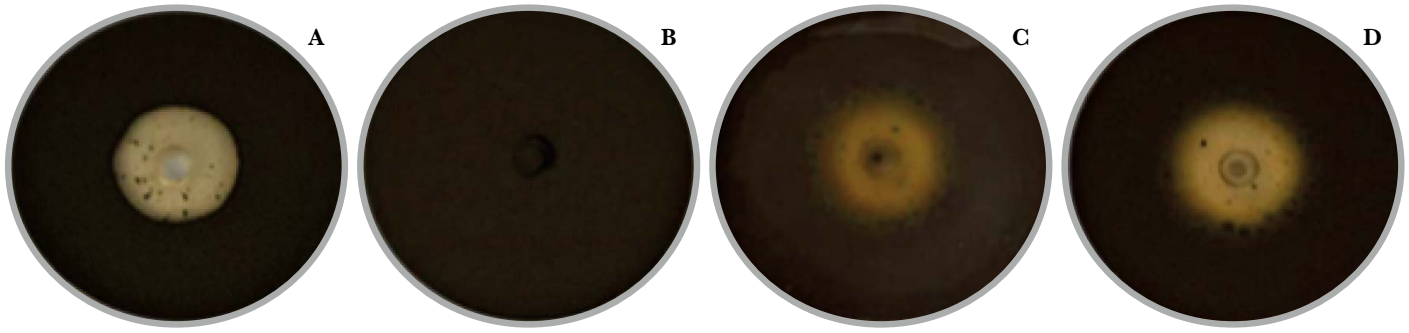

Figure 1. Antifungal activity evaluation of hydroethanolic extract of *D. muscipula* against *P. fijiensis*. A) Positive control (Neomycol®); B) Negative control (ethanol-water); C) D. muscipula *in vitro*; D) acclimatized *D. muscipula*.

sporulation of fungal pathogens. Mosquera *et al.* (2009) reported inhibition of 100% of *P. fijiensis*, using methanolic extracts of topobea (*Topobea* cf discolor), which is associated to the high concentration of secondary metabolites, among which the flavonoids were found, which have been reported in *D. muscipula* J. Ellis. Meanwhile, Jiménez *et al.* (2003) describe that a greater exposure to environmental conditions can induce the proliferation of secondary metabolites. Kreher *et al.* (1990) report the isolation of naphthoquinones: plumbagin, hydroplumbagin 4-O-β-glucopyranoside, 3-cloroplumbagin and droserone from methanol extracts of *D. muscipula* plants. Eilenberg *et al.* (2010) report the antifungal activity of droserone and 5-O-metyldroserone in the secretions of the traps of the *Nepenthes khasiana* carnivore plant, with the inhibition of spore germination and the growth of plant pathogens such as *Botrytis cineria*, *Rhizoctonia solani*, *Fusarium oxysporum* and *Mycosphaerella graminícola*, as well as the inhibition of human pathogen fungi growth such as *Candida albicans* CBS 562, *C. albicanmas*, *C. krusei*, *C. glabrata*, *Aspergillus flavus*, *A. niger*, *A. fumiga*. This suggests that the antifungal activity observed in this study is related to the presence of secondary metabolites in the hydroethanolic extracts of *D. muscipula*.

CONCLUSIONS

The hydroethanolic extracts of *in vitro* and acclimatized plants of *Dionaea muscipula* J. Ellis presented bacteriostatic activity against *Escherichia coli* and *Bacillus cereus*. However, they did not show antifungal activity against *Aspergillus niger* and *Fusarium oxysporum*. However, they showed inhibition of fungal growth of *Pseudocercospora fijiensis*.

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