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Alternative Use of Extracts of Chipilín Leaves (*Crotalaria longirostrata* Hook. & Arn) as Antimicrobial

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Abstract: The genus Crotalaria comprises about 600 species that are distributed throughout the tropics and subtropical regions of the world; they are antagonistic to nematodes in sustainable crop production systems, and have also shown antimicrobial capacity. Chipilín (*C. longirostrata*), which belongs to this genus, is a wild plant that grows in the state of Chiapas (Mexico) and is traditionally is used as food. Its leaves also have medicinal properties and are used as hypnotics and narcotics; however, the plant has received little research attention to date. In the experimental part of this study, dried leaves were macerated by ethanol. The extract obtained was fractionated with ethyl ether, dichloromethane, ethyl acetate, 2-propanone, and water. The extracts were evaluated against three bacteria—namely, *Escherichia coli* (Ec), *Citrobacter freundii* (Cf), and *Staphylococcus epidermidis* (Se)—and three fungi—*Fusarium oxysporum* A. comiteca (FoC), *Fusarium oxysporum* A. tequilana (FoT), and *Fusarium solani* A. comiteca (FSC). During this preliminary study, a statistical analysis of the data showed that there is a significant difference between the control ciprofloxacin (antibacterial), the antifungal activity experiments (water was used as a negative control), and the fractions used. The aqueous fraction (WF) was the most active against FoC, FsC, and FoT (30.65, 20.61, and 27.36% at 96 h, respectively) and the ethyl ether fraction (EEF) was the most active against Se (26.62% at 48 h).

Keywords: traditional food; antimicrobial; bioassay; PIRG; fractions

1. Introduction

The number of plant diseases caused by pests attacking crops has increased the need for new antimicrobials to eliminate the pathogens. This need has led to a renewed focus on natural extracts from plants, fungi, bacteria, algae, etc. [1]. Every year, plant diseases cause an estimated 40 billion dollars in losses worldwide [2]. Chemical fungicides are not readily biodegradable and tend to persist for years in the environment. As a result, the use of natural products for the management of fungal diseases in plants is considered a reasonable substitute for synthetic fungicides [3]. The genus Crotalaria includes around 600 species distributed throughout the tropics and subtropical regions of the world, which have been used as antagonists to nematodes in sustainable crop production systems [4,5].

There are also previous studies showing the anti-inflammatory [6], anthelmintic [7], antitumoral capacity [8] and antimicrobial activity of *C. madurensis* [9] and *C. burhia* [10,11], which showed activity against *Bacillus subtilis* and *Staphylococcus aureus*, while *C. pallida* demonstrated that it has an effect on *Escherichia coli* and *Pseudomonas* sp. [12–14]. The species of this genus contain alkaloids, saponins, and flavonoids to which biological activity is attributed [4]. Chipilín (*Crotalaria longirostrata*) belongs to this genus; it is a wild plant that grows in the state of Chiapas, Mexico that is used traditionally food [15], and also has ethnobotanical properties as hypnotics and narcotics [16]. Since there are few reports of the biological activity of the species *C. longirostrata*, this study fractionates the crude extract from Chipilín (*C. longirostrata*) leaves, obtaining ethyl ether (FEE), dichloromethane (FDM), ethyl acetate (FEA), 2-propanone (FAO), and aqueous fractions (FW), as a preliminary measure in order to evaluate its potential as an antimicrobial.

2. Materials and Methods

2.1. Plant Material

The leaves of *C. longirostrata* were collected in Ocozocoautla, Chiapas, México, geographic location: latitude 16°45′32′′ N and longitude 93°21′53′′ O.

2.2. Extraction

The plant material were shade-dried for seven days. The dried leaves were grounded to a fine texture, then soaked (0.15 g of dry matter/mL of solvent) in EtOH (96%) (Meyer, CDMex, Mexico) for 15 days. After filtration, the extract was evaporated to obtain the crude extract. About half of the crude extract was suspended in distilled water (H_2O) (Sigma-Aldrich-Merck, Darnstadt, Germany) and separately partitioned with ethyl ether (Et_2O) (Meyer, CDMex, Mexico), followed by dichloromethane (CH_2Cl_2) (Meyer, CDMex, Mexico), ethyl acetate (AcOEt) (Meyer, CDMex, Mexico), and 2-propanone (C_3H_6O) (Meyer, CDMex, Mexico), respectively. The organic layer of each solvent was concentrated to dryness under reduced pressure, and dried over anhydrous sodium sulfate to afford Et_2O, CH_2Cl_2, AcOEt, C_3H_6O, and H_2O fractions. These fractions were stored at 4 °C until use. Each fraction was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich-Merck, Darnstadt, Germany), and prepared at a concentration of 200 mg/mL in all bioassays [17].

2.3. Cultivation of Microorganism

The microorganisms used were: *Escherichia coli* (Ec) (ITTG-1879), *Citrobacter freundii* (Cf) (Cf-ITTG), and *Staphylococcus epidermidis* (Se) (ITTG-850), which were inoculated on Tryptone-Soya-Agar TSA and incubated at 33 ± 2 °C for 24 h [18]; and *Fusarium oxysporum* A. comiteca (FoC) (FoC-ITTG), *Fusarium oxysporum* A. tequilana (FoT) (FoT-ITTG), and *Fusarium solani* A. comiteca (FSC) (FsC-ITTG), which were inoculated in potato dextrose agar (PDA) at 28 ± 2 °C. For the bioassays, Whatman No. 1 paper discs of 6 mm diameter were placed on the periphery of the Petri dishes [18].

2.4. Evaluation of Antifungal Activity

In order to evaluate the effect by direct contact of the fractions on the microorganisms, Whatman No. 1 paper discs were impregnated with 10 µL of the corresponding fraction. Later, discs were placed with the microorganism on the disks with the fraction. In the second bioassay, the effect of the fractions that showed antimicrobial activity in the first bioassay was evaluated. A 5 mm paper disc with the fraction was placed in the Petri dishes colonized by the microorganism. Microbial growth was measured every 24 h until 96 h, as a positive control sterile distilled water (H_2O) was employed. A solvent test was also performed using a filter paper disc treated with sterile DMSO [19]. Negative control test wity DMSO were performed (data provided in the Supplementary Materials). The diameters for the inhibition zones were measured in millimeters. The percentage inhibition of

radial growth (PIRG) was calculated using the Abbott formula: PIRG (%) = [(RC − RT)/RC] × 100, where RC is the radius of the control, and RT the radius of the treatment [20].

2.5. Evaluation of Antibacterial Activity

A volume of 0.1 mL of inoculated cell suspension broth was placed on each Petri dish (Ec 2.95 × 10^3 CFU/mL; Cf 8.47 × 10^3 CFU/mL and 2.86 × 10^6 CFU/mL for Se) [12]. Then, four Whatman No. 1 paper discs were impregnated with 10 µL of the corresponding fraction. The diameter of the growth inhibition zone was measured at 15 h, 24 h, 40 h, and 48 h. Ciprofloxacin 125 mg/mL for Ec and Cf, and chloramphenicol at 5 mg/mL for Se [20] were used as a positive control. A solvent test was also performed using a filter paper disc treated with sterile DMSO [19]. DMSO tests were performed, observing growth on the whole plate identical to the control (water) Percentage inhibition (PI) was calculated using a modified expression of the Abbott formula: PI (%) = [DT/DC] × 100, where DC is the diameter of the inhibition halo of the control, and DT is the diameter of the inhibition halo of the treatment [20].

2.6. Experimental Design

A completely randomized experimental design with three replicates was used for each microorganism, taking as a response variable to PIRG or PI. A simple ANOVA was performed with a comparison of means using the Tukey test at 95% confidence.

3. Results

3.1. Evaluation of Antifungal Activity

The bioassays were carried out to know the possible antimicrobial activity of the different fractions, and directed towards the most promising fraction after other specific bioassays.

In the first bioassay, the fractions showed a fungistatic effect on the three fungi. For each fungus, the most effective fraction at 24 h was different. In the case of FoC, it was the aqueous fraction with a PIRG of 50.00%. For FoT, the highest value of PIRG was obtained with the dichloromethane fraction (FDM, 61.76%), and for FsC, it was the 2-propanone fraction that obtained higher values of inhibition, with 35.00% (Table 1). However, for the three species fungi, the aqueous fraction (FW) was the one with the highest percentage of inhibition (PIRG) at 48 h, 72 h, and 96 h (Table 1).

For the second bioassay, the aqueous fraction was employed. For FoC and FsC, a mycelial growth-promoting effect was observed at the end of the test time. For FoT, the aqueous fraction showed a value of 27.94% of inhibition in the first 24 h; however, this effect did not last after 72 h (Table 2).

3.2. Evaluation of Antibacterial Activity

The fractions for 2-propanone (AF) and ethyl acetate (EAF) had a low antimicrobial activity at 15 h (17.62% and 18.10%, respectively). The lowest percentage inhibition was observed in the dichloromethane fraction (5.71% at 15 h); while the ethyl ether fraction (EEF) showed better antimicrobial activity than the other fractions at 48 h (26.62%) against Se (Table 3).

Table 1. Percent inhibition by direct contact of Chipilín (*C. longirostrata*) active fractions on phytopathogenic fungal species.

Treatment	Strain											
	FoC				FoT				FsC			
	Time (h)											
	24	48	72	96	24	48	72	96	24	48	72	96
(SDW)	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{ab}	0.00 ^{ab}	0.00 ^{a}	0.00 ^{a}
AF	25.00 ^{ab}	14.13 ^{b}	4.91 ^{ab}	9.61 ^{bc}	49.02 ^{bc}	8.99 ^{ab}	6.92 ^{b}	18.66 ^{b}	35.00 ^{b}	11.61 ^{ab}	7.29 ^{a}	8.65 ^{ab}
EAF	40.00 ^{b}	28.26 ^{c}	17.89 ^{c}	13.25 ^{c}	57.84 ^{bc}	12.70 ^{b}	9.34 ^{b}	15.42 ^{b}	7.50 ^{ab}	12.26 ^{b}	10.42 ^{ab}	12.98 ^{bc}
WF	50.00 ^{b}	41.85 ^{d}	26.67 ^{d}	30.65 ^{d}	40.20 ^{b}	16.40 ^{b}	18.34 ^{c}	27.36 ^{c}	2.50 ^{ab}	15.48 ^{b}	20.83 ^{b}	20.61 ^{c}
DMF	42.50 ^{b}	20.65 ^{bc}	8.77 ^{b}	12.99 ^{c}	61.76 ^{c}	12.70 ^{b}	8.30 ^{b}	18.16 ^{b}	−17.50 ^{a}	9.68 ^{ab}	8.68 ^{a}	10.69 ^{bc}
EEF	42.50 ^{b}	18.48 ^{bc}	10.18 ^{bc}	3.38 ^{ab}	55.88 ^{bc}	2.12 ^{a}	3.46 ^{ab}	15.92 ^{b}	22.50 ^{b}	−5.16 ^{a}	10.76 ^{ab}	13.99 ^{bc}

The data are given in percentage inhibition of radial growth (PIRG). FoC (*Fusarium oxysporum* A. comiteca), FoT (*Fusarium oxysporum* A. tequilana), and FSC (*Fusarium solani* A. comiteca). AF: 2-propanone fraction; EAF: ethyl acetate fraction; WF: aqueous fraction; DMF: dichloromethane fraction; EEF: ethyl ether fraction and SDW: sterile distilled water. Latin letters a,b and c,d (superscript) in the same column indicates significant differences. Tukey 95% $p \leq 0.0046$.

Table 2. Volatility test in the aqueous fraction of Chipilín (*C. longirostrata*) on phytopathogenic fungal species.

Treatment	Strain								
	FoC			FoT			FsC		
	Time (h)								
	24	48	72	24	48	72	24	48	72
(SDW)	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}	0.00 ^{a}
WF	−2.00 ^{a}	−7.09 ^{b}	−5.53 ^{b}	27.94 ^{b}	4.44 ^{a}	−4.78 ^{a}	3.77 ^{a}	−6.47 ^{b}	−5.63 ^{b}

The data are given in PIRG. FoC (*Fusarium oxysporum* A. comiteca), FoT (*Fusarium oxysporum* A. tequilana), and FSC (*Fusarium solani* A. comiteca). WF: aqueous fraction and SDW: sterile distilled water. Latin letters a,b (superscript) in the same column indicates significant differences. Tukey 95% $p \leq 0.0529$.

Table 3. Percent inhibition of Chipilín (*C. longirostrata*) fractions on pathogenic bacteria.

Treatment	Strain											
	Ec				Cf				Se			
	Time (h)											
	15	24	40	48	15	24	40	48	15	24	40	48
Positive control	100 ^{a}				100 ^{c}	100 ^{b}	100 ^{b}	100 ^{c}	100 ^{c}	100 ^{d}	100 ^{d}	100 ^{d}
AF	NI				NI				17.62 ^{b}	13.78 ^{b}	13.97 ^{b}	14.39 ^{b}
EAF	NI				5.43 ^{a}	3.43 ^{a}	1.43 ^{a}	0.00 ^{a}	18.10 ^{b}	5.61 ^{a}	6.15 ^{ab}	5.04 ^{a}
WF	NI				NI				10.48 ^{a}	8.67 ^{ab}	8.38 ^{ab}	5.04 ^{a}
DMF	NI				NI				5.71 ^{a}	4.08 ^{a}	3.91 ^{a}	2.16 ^{a}
EEF	NI				10.29 ^{b}	4.00 ^{a}	4.00 ^{a}	2.59 ^{b}	24.29 ^{b}	22.45 ^{c}	22.91 ^{c}	26.62 ^{c}
MSD	-				3.82523	3.46982	2.57792	1.50361	7.07667	7.63128	7.9051	7.60449

The data are given in percentage inhibition (PI). Ec (*Escherichia coli*), Cf (*Citrobacter freundii*), and Se (*Staphylococcus epidermidis*). AF: 2-propanone fraction; EAF: ethyl acetate fraction; WF: aqueous fraction; DMF: fraction of dichloromethane; EEF: ethyl ether fraction; and positive control ciprofloxacin at 125 mg/mL for *E. coli* and *C. freundii*, and chloramphenicol at 5 mg/mL for *S. epidermidis*. NI: not inhibited. Latin letters a,b,c,d (superscript) in the same column indicates significant differences. Tukey 95% $p \leq 0.0000$. Minimum Significant Difference (MSD).

4. Discussion

In the last two decades, there has been growing interest in research for extracts for medicinal plants as sources of new antimicrobial agents [12–14]. Recent findings about species of the genus *Crotalaria* describe their biological activity, by example the ethanolic fractions of *C. retusa*, the chloroform fraction of *C. prostrate*, the ethanolic extract of *C. medicaginea*, the ethanolic extract of *C. pallida*, the methanolic extract of *C. burhia*, and the fractions of *C. bernieri* and *C. madurensis* showed inhibitory capacity against *E. coli* [4,9,10,13,21]. The species *C. longirostrata* has been reported to have ethnobotanical activity, but an antimicrobial evaluation had not yet been done, and the chemical compounds responsible remained unknown. We evaluated its antimicrobial potential, finding that the fraction of dichloromethane is more effective in inhibiting the growth of FoT (61.7%) in comparison with the aqueous extract of *C. medicagenina* (33%) and the methanolic extract of *C. filipes* (55%). However, it was less efficient than the isolated peptide of *C. pallida* (70%) [1,12,22]. Further, the aqueous fraction showed low inhibition values (2.5%) against *F. solani*, while the 2-propanone fraction (35%) revealed activity for the aqueous extract of *C. juncea* [23]. Subsequently, the antibacterial evaluation of the fractions of *C. longirostrata* found statistically significant differences between the fractions and the control in *C. freundii* and *S. epidermidis*; however, the percentage of inhibition was lower than that of the antibiotic (control).

The results obtained of this preliminary study show the fungistatic capacity, but not fungicidal capacity, of the fractions obtained from the Chipilín (*C. longirostrata*). Some phenolic compounds, alkaloids, essential oils, and glycosides have shown to be responsible for antifungal activity [1]. This suggests the presence of these compounds in the fractions that were analyzed, which proved to be more effective against fungi than bacteria. The effect of substances of plant origin is due to mechanisms of direct fungitoxic action [21], while the bactericidal potential is associated with anthraquinones and

flavonoids of a catechinic nature [24]. There is a great diversity in the forms of action of secondary metabolites that have been reported as antifungal [25–27]. However, each extract showed a spectrum of specific activity that could be due to the difference between the chemical nature and the concentration of bioactive compounds in extracts [21]. For example, the EEF fraction against FsC showed low inhibition or equal to the growth of the control, so its PIRG value was diminished. Therefore, it is necessary to carry out further phytochemical studies for the identification of the secondary metabolites of the Chipilín (*C. longirostrata*) leaves that are responsible for its antimicrobial activity.

Supplementary Materials: The following are available online at http://www.mdpi.com/2071-1050/10/3/883/s1, Table S1. Solvent test on the mycelial growth of the phytopathogenic fungi evaluated.

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Author Contributions: Selene Lagunas-Rivera conceived and designed the experiment and all authors were involved in analyzing the data; Johana Miranda-Granados and Cesar Chacon performed the experiments and analyzed all the samples; Nancy Ruiz-Lau, Peggy Alvarez-Gutierrez and Rocio Meza-Gordillo contributed analysis tools; Maria Elena Vargas-Diaz and Gerardo Zepeda-Vallejo contributed with the reagents/materials/analysis tools, all authors were involved and contributed to writing the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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