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Pre-emergent bioherbicidal activity of creosote bush and tarbush extracts against buffalo gourd

Actividad bioherbicida pre-emergente de extractos de gobernadora y tarbush sobre calabacita loca

Lluvia de Abril Alexandra Soriano-Melgar[1,2*], Grecia Estefanía Martínez-Sosa[1], Martha Monzerrath Orozco-Sifuentes[1], Juan Alberto Ascacio-Valdés[1], Lissethe Palomo-Ligas[1], Lizeth Guadalupe Campos-Muzquiz[1]

[1]Facultad de Ciencias Químicas, Universidad Autónoma de Coahuila. Blvd. Venustiano Carranza 935, Colonia República, CP. 25280. Saltillo, Coahuila de Zaragoza, México.

[2]Programa de Investigadoras e Investigadores por México, SECIHTI. Insurgentes Sur, 1582, Crédito Constructor, CP. 03940. Ciudad de México, México.

*Correspondence author: lluviasoriano_fcq@uadec.edu.mx

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ABSTRACT. Effective, safe weed control is crucial for sustainable food production. Bioherbicides derived from plant extracts present a promising alternative. Extracts from plants like creosote bush (*Larrea tridentata*) and tarbush (*Flourensia cernua*) contain allelopathic compounds that can disrupt weed germination and growth. The aim of this study was to evaluate the effect of *L. tridentata* and *F. cernua* extracts on pre-emergent control of buffalo gourd (*Cucurbita foetidissima*). Plant extracts were prepared using microwave and ultrasound technologies with "green" solvents and were applied at different doses [3 (low), 6 (medium), and 12 (high) L of extracts in 200 L per hectare] on *C. foetidissima* seeds, and their effect on germination, seedling growth, dry biomass, and mechanisms of action [on photosynthetic pigments (chlorophylls a, b, total, and carotenoids), total proteins, antioxidant enzymes (catalase and peroxidase), total phenolic compounds, antioxidant capacity, and oxidative stress (lipid peroxidation)] were evaluated. The phytochemicals like quercetin and (+)-gallocatechin in the bioherbicide extract were found. The high dose of the extract decreased germination rates and decreased seedling growth in *C. foetidissima* compared to the control. Although dry biomass showed no significant change, a trend toward reduction was observed in treated seedlings. Chlorophyll and carotenoid levels decreased in all treatments, while protein levels were unaffected. Low-dose treatments decreased catalase activity, and the high dose increased lipid peroxidation. This study demonstrates that allelopathic plant extracts effectively inhibit *C. foetidissima* seedling growth, with compound concentration and content critical to bioherbicide effectiveness.
Keywords: Allelopathic compounds, natural extracts, mechanisms of action, oxidative stress, weed control.

RESUMEN. Los métodos de control de malezas seguros y efectivos son esenciales para una producción sostenible. Los bioherbicidas de extractos de plantas, como gobernadora (*Larrea tridentata*) y hojasén (*Flourensia cernua*), ofrecen una alternativa prometedora, ya que contienen compuestos alelopáticos que interrumpen la germinación y el crecimiento de malezas. Este estudio evaluó el efecto de los extractos de *L. tridentata* y *F. cernua* en el control pre-emergente de la calabaza loca (*Cucurbita foetidissima*). Se prepararon los extractos usando microondas y ultrasonido con solventes "verdes" y se aplicaron en distintas dosis [3 (baja), 6 (media) y 12 (alta) L de extractos en 200 L por hectárea] sobre semillas de *C. foetidissima*. Se evaluaron sus efectos en germinación, crecimiento de plántulas, biomasa seca y mecanismos de acción [pigmentos fotosintéticos, proteínas, enzimas antioxidantes (catalasa y peroxidasa), compuestos fenólicos, capacidad antioxidante y estrés oxidativo]. Se identificaron fitoquímicos como quercetina y (+)-galocatequina en los extractos. La dosis alta redujo las tasas de germinación y el crecimiento de las plántulas de *C. foetidissima* en comparación con el control. Aunque la biomasa seca no mostró un cambio significativo, se observó una tendencia a la reducción en las plántulas tratadas. Los niveles de clorofila y carotenoides disminuyeron en todos los tratamientos, mientras que las proteínas no se vieron afectadas. La dosis baja redujo la actividad de la catalasa, y la dosis alta aumentó la peroxidación lipídica. El estudio demuestra que los extractos de plantas alelopáticas inhiben el crecimiento de *C. foetidissima*, siendo la concentración y el contenido de los compuestos esenciales para su eficacia.
Palabras clave: Compuestos alelopáticos, extractos naturales, mecanismos de acción, estrés oxidativo, control de malezas.

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INTRODUCTION

The world population continues to grow and agricultural land availability decreases, as indicated by projections from the Food and Agriculture Organization (FAO), which suggest a significant increase in food demand by the year 2050 (FAO 2009). With the increasing global population and constant pressure on natural resources, it is important to adopt innovative and sustainable approaches to guarantee food security and protect the environment (Nusrat *et al*. 2018). The constant growth in agricultural demand presents a compelling need to find effective methods to address the major challenges in agriculture, which faces constant challenges, with one of the most pressing being the effective, safe, and sustainable control of weeds (Hasan *et al*. 2021). Despite their contribution to plant diversity in natural ecosystems, weeds represent a significant challenge in agricultural production due to their ability to reduce crop yield and quality, host of pathogens and pests of agricultural importance, and increase production costs, as they compete with main crops for resources such as nutrients, water, sunlight, space and interferes with harvest (Siyar *et al*. 2019, Mehdizadeh and Mushtaq 2020, Roberts *et al*. 2022).

Weeds are even considered a considerable threat to agricultural production, surpassing other pests in their ability to reduce crop yields (Khamare *et al*. 2022); as weeds can decrease crop yields by over 30% (Sidhu *et al*. 2023, Gao and Su 2024). Although these losses cannot be attributed to a single weed species but to different species (Kostina-Bednarz *et al*. 2023). Buffalo gourd (*C. foetidissima* Kunth) is a very common perennial herbaceous plant, considered a weed, related to domesticated species of cucurbits, distributed in arid and semi-arid regions of North and Central America, it can grow in places between 1 600 and 2 500 m above sea level, adapted to drought, high temperatures, and biotic stress, with some uses in cosmetics (Estrada-Castillón *et al*. 2014, Mejía-Morales *et al*. 2021), it is susceptible to different viruses that can easily spread with the proximity of other uninfected plants (Rosemeyer *et al*. 1986).

Effective control of weeds is achieved through various methods, including cultural, mechanical, biological, and chemical approaches (Wazir *et al*. 2011, Khamare *et al*. 2022, Parida and Deb 2023, Gao and Su 2024). Traditionally in modern agriculture, the use of synthetic chemical herbicides for weed control has been widely increased (Wazir *et al*. 2011). The extensive and indiscriminate use of these products has raised significant concerns in terms of negative impact to environmental, and human health, and weed resistance development, raising concerns about food security, underscoring the need to develop safer and more sustainable alternatives, such as bioherbicides (Nusrat *et al*. 2018, Anupama *et al*. 2023, Kostina-Bednarz *et al*. 2023).

Bioherbicides are mainly derived from plant extracts, which have emerged as a promising alternative to address the challenges associated with synthetic herbicides (Khamare *et al*. 2022, Shadab *et al*. 2024). Among the main allelochemical compounds obtained from plants with potential to be used as bioherbicides are phenols (including flavonoids, lignins, tannins, benzoic acid and its derivatives), terpenoids, long-chain fatty acids, organic cyanides, aldehydes, organic acids, ketones, lactones, and nitrogen-containing compounds (such as alkaloids) (Parida and Deb 2023, Motmainna *et al*. 2023, Arora *et al*. 2024). The phytotoxicity of these allelochemicals depends on quantity and composition, environmental factors, and management practices (Arora *et al*. 2024), but the mode of action of some allelochemicals remains unknown (Khamare *et al*. 2022). These

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compounds interfere with plant growth and germination processes, protein synthesis along with their action on antioxidant enzymes (such as peroxidases) and oxidative stress, inhibition of components of photosynthesis and mitochondrial respiration; highlighting the importance of understanding these processes for the development of new weed control strategies (Kostina-Bednarz *et al*. 2023, Parida and Deb 2023).

Bioherbicides, by harnessing secondary metabolites and phytochemicals from plants, offer a safer, environmentally friendly solution for weed control compared to conventional chemical herbicides (Nusrat *et al*. 2018). However, despite advantages like lower toxicity and greater host specificity, challenges remain in their development and application. These challenges include host specificity, toxicity, environmental influences, formulation processes, commercialization, identification of suitable active ingredient sources, and understanding different modes of action, which require ongoing research (Roberts *et al*. 2022, Arora *et al*. 2024). Current research aims to better understand allelopathic interactions to develop herbicides derived from allelochemicals that are effective and environmentally safe (Siyar *et al*. 2019).

Creosote bush [*Larrea tridentate* (DC.) Coville] and tarbush (*Flourensia cernua* DC.) have emerged as plants of interest for their allelopathic properties (Hyder *et al*. 2002). *L. tridentata* is a perennial shrub in Mexico and the USA, rich in bioactive compounds, mainly phenolics in stems and green leaves (Hyder *et al*. 2002), including nordihydroguaiaretic acid, ellagic acid, gallic acid, tannins, catechins, and quercetin, which possess antioxidant, antifungal, and insecticidal properties with high potential for various industrial applications (DeLoach *et al*. 1986, Morales-Ubaldo *et al*. 2022). *F. cernua*, found in arid and semi-arid regions of Mexico and adjacent areas of the United States, is considered a pasture weed that contains a variety of phytochemicals, where at least 102 compounds have been identified, including phenolics like flavonoids, terpenoids, alkaloids, lactones, saponins, terpenes, condensed tannins, flavonoid glycosides, luteolin 7-rutinoside, and apigenin-6-C-glucosyl-8-carabinoside, known for antifungal and antioxidant activities, and potential use as natural emulsion stabilizers (DeLoach *et al*. 1986, De León-Zapata *et al*. 2021, Linares-Braham *et al*. 2023, Da-Silva-Carvalho *et al*. 2023). Thus, it is essential to evaluate allelochemicals as natural herbicides, including their modes of action and specific interactions, to achieve the effectiveness of synthetic herbicides (Khamare *et al*. 2022). The goal of this project was to investigate the effects of creosote bush (*L. tridentata*) and tarbush (*F. cernua*) extracts obtained using microwave and ultrasound technologies on controlling *C. foetidissima* as a potential pre-emergent bioherbicidal extract.

MATERIALS AND METHODS

Plant material

Plants of creosote bush (Figure 1A) and tarbush (Figure 1B) were collected at coordinates 25°18'30.5 N, 101°04'21.8 W, in the region known as La Encantada, Coahuila de Zaragoza, Mexico. The creosote bush and tarbush plants were herbarized and recorded in the herbarium (104701 and 104702, respectively) of the Universidad Autónoma Agraria Antonio Narro (UAAAN). The plant samples were air-dried at room temperature and forced air, leaves were separated, pulverized, followed by grinding and sieving (mesh No. 100) with a particle size smaller than 150 µm. Extracts were obtained using simultaneously microwave and ultrasound, employing 70% (v/v) ethanol as the extracting agent and creosote bush (leaves), tarbush (leaves), pecan shells (*Carya illinoinensis*, solid waste), and eucalyptus (*Eucalyptus* sp. leaves) (56, 19, 12.5, and 12.5%, respectively). Therefore, the main components of the bioherbicide, in majority proportion, are related to the compounds of creosote bush/tarbush extracts. The addition of walnut shell and eucalyptus leaf extract was made to improve the formulation (previously developed within the work group) (Castillo-Reyes *et al*. 2022). The extraction conditions, as well as other components and details of the extract, are part of a pending patent. Seeds of the *C. foetidissima* were donated by researchers from the Seed Bank of the UAAAN. Weed seeds of *C. foetidissima* were washed with running water and commercial detergent to remove field impurities. Subsequently, they were subjected to water imbibition for 48 h to induce germination.

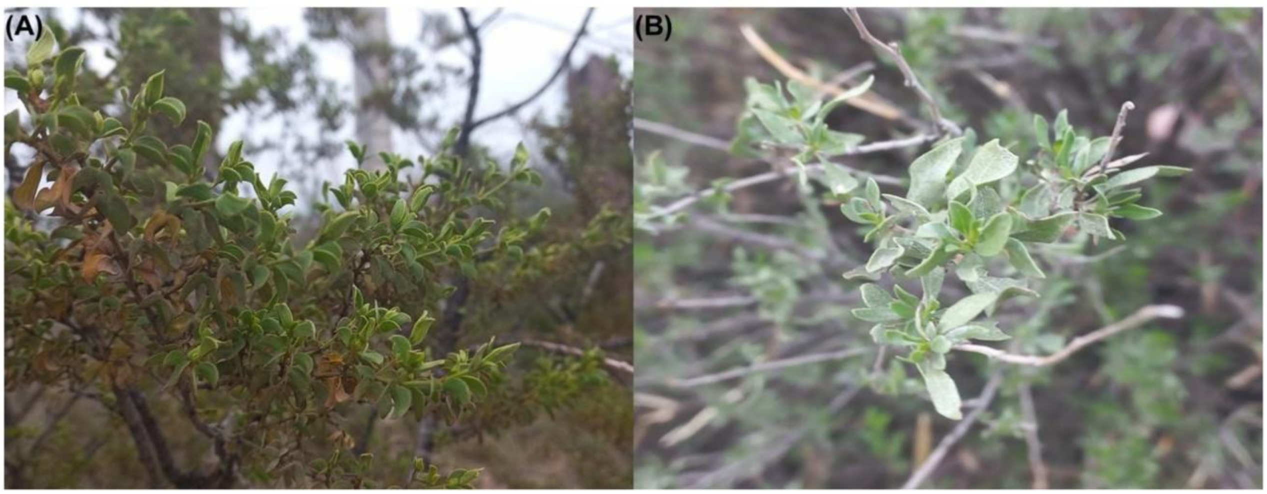

Figure 1. (A) Creosote bush (*Larrea tridentata*) and (B) tarbush (*Flourensia cernua*) plants collected.

Phytochemical characterization of plant extracts by HPLC-ESI-MS

Plant extracts were analyzed using an HPLC-ESI-MS system equipped with an autosampler (Varian ProStar 410, USA), a ternary pump (Varian ProStar 230I, USA), and a PDA detector (Varian ProStar 330, USA), alongside a liquid chromatography mass spectrometer (Varian 500-MS IT Mass Spectrometer, USA) equipped with an electrospray ionization (ESI) source (Ascacio-Valdés *et al.* 2016). Samples (5 µL) were injected into a Denali C18 column (150 mm × 2.1 mm, 3 µm) at 30 °C. The analysis was performed using a mobile phase consisting of 0.2% formic acid (solvent A) and acetonitrile (solvent B), with a gradient ranging from 3% B to 50% B over a period of 45 min. The flow rate was set at 0.2 mL min^{-1}, and detection wavelengths were 245, 280, 320, and 550 nm. The effluent was directed into the mass spectrometer operating in negative ionization mode [M-H]-1, using nitrogen and helium as support gases. Ionization parameters included a spray voltage of 5.0 kV and a temperature of 350 °C. Data were collected and processed using MS Workstation software, with a full scan acquired over the m/z range of 50-2000 (Ascacio-Valdés *et al*. 2016).

Treatments with bioherbicidal extracts

Polystyrene domes (two thirds were covered by the substrate) with PeatMoss commercial substrate sterilized previously were prepared and moistened with distilled water to saturation (pH of 5.8-6.1

and an electrical conductivity of 0.5-0.7 dS m^{-1}). Sixteen *C. foetidissima* seeds per dome were placed in triplicate. Treatments (bioherbicidal extracts) were applied as spray at increasing doses, where dilutions equivalent to field-applied doses of 3 (low dose), 6 (medium dose), and 12 (high dose) L of extracts in 200 L of bioherbicide per hectare (these equivalent doses were calculated based on the surface area of the polystyrene domes used in the experiment) were performed, and water spray was used as a control. Only a single dose was applied as pre-emergence treatments, and the domes were irrigated with a volume of 10 mL of distilled water per week.

Germination and growth

Germination percentage: The number of germinated seeds was evaluated every third day for 45 days relative to the total number of seeds placed per dome. Results were reported only at days 0, 4, 21, and 45. The germination percentage (%G) was calculated using the following equation: %G = (GS/TS) * 100. Where, GS = Number of germinated seeds, TS = Total number of seeds.

Shoot growth: Using a digital Vernier caliper (Caliper Accuracy, China), the length of plant shoots was measured after their development (45 days after the initial application of treatments), and results were reported in mm. Due to the roots being very fragile and breaking when removing the substrate, their measurement was not considered to avoid bias.

Dry biomass: Using an analytical balance (0.0001, Ohaus, E-02130, USA), the fresh tissue of 3 seedlings (roots and shoots separated) was weighed per triplicate, then dried at 60 °C for 48 h until a constant weight was achieved, and the tissue was weighed again. The percentage of dry biomass (%DB) was calculated using the following equation: %DB = [1 – (Iw-Fw)/Pi]*100. Where, Iw = Initial weight, Fw = Final weight.

Mechanisms of action

Photosynthetic pigments and protein: To determine the content of chlorophylls a, b, and total, the method described by Sumanta *et al*. (2014) modified for microplate was used. 0.15 g of plant tissue was weighed, homogenized with 1.5 mL of methanol (80%, v/v) for 15 s in a homogenizer (Fastprep-24, MP Biomedicals, USA), the homogenized sample was centrifuged (Select BioProducts, Select Spin 17R, USA) at 3 000 *x g* for 15 min at 4 °C. Then, 200 µL of sample was placed in microplate per replicate, and absorbance was read at 652.4, 665.2, and 470 nm (BioTek Instruments, Epoch, USA). Chlorophyll content was calculated to report results in µg of pigment g^{-1} of fresh weight (FW).

To determine the total protein content, the method described by Bradford (1976) modified for microplate was used. An enzymatic extract was obtained from 100 mg of plant tissue, 1 mL of 100 mM phosphate buffer, pH 7, and 30 mg of polyvinylpyrrolidone (PVP). The mixture was centrifuged (Select BioProducts, Select Spin 17R, USA) at 3 000 *x g* for 15 min at 4 °C. The reaction mixture consisted of 155 µL of distilled water, 45 µL of Bradford colorant, and 25 µL of enzymatic extract. Absorbance was determined at 620 nm in a microplate reader (BioTek Instruments, Epoch, USA). A standard curve of bovine serum albumin (BSA) from 0-0.2 mg mL^{-1} was used to report results as mg protein g^{-1} FW.

Antioxidant enzymes (catalases and peroxidases): The previously described enzymatic extract was used, and the reaction mixture consisted of 10 µL of extract with 990 µL of hydrogen peroxide (H_2O_2, 20 mM). Solutions were kept at 25 °C, and samples on ice. Absorbance was read at 240 nm (Varian Cary50 UV–visible, Spectralab Scientific Inc. Canada) every 20 s for 2 min. One unit of catalase is defined as the amount of enzyme needed to reduce 1 µmol of H_2O_2 min^{-1}, and results were reported as UCAT mg^{-1} protein.

For determination of total peroxidases (Kar and Mishra 1976), the reaction mixture consisted of 675 µL of water, 62 µL of phosphate buffer (100 mM, pH 7), 13 µL of peroxide (H_2O_2, 3%), 250 µL of pyrogallol (50 µMoles), and 250 µL of enzymatic extract. Absorbance was read at 420 nm (Varian Cary50 UV–visible, Spectralab Scientific Inc. Canada) every 20 s for 2 min, and results were reported as UPOX mg^{-1} protein.

Oxidative stress: To determine the content of total phenolic compounds, the method described by Singleton and Rossi (1965) was used. An extract was obtained from 0.1 g of plant tissue with 1 mL of 80% methanol. 20 µL of the sample was mixed with 100 µL of Folin-Ciocalteu reagent (diluted with water 1:10 v/v) and incubated for 1 min. 80 µL of sodium carbonate (7.5%, w/v) was added, and incubated for 30 min at room temperature. Absorbance was measured at 765 nm in a microplate reader (BioTek Instruments, Epoch, USA).

The commercial free radical DPPH (2,2-diphenylpicrylhydrazyl) was used to determine antioxidant capacity (Brand-Williams *et al*. 1995). An extract was generated from 0.1 g of plant tissue with 1 mL of methanol (80%, v/v). The reaction mixture consisted of 50 µL of the sample and 150 µL of DPPH (125 µM), which was incubated at room temperature for 15 min, and absorbance was measured at 515 nm in a microplate reader (BioTek Instruments, Epoch, USA). A standard curve of gallic acid (0-20 µMoles mL^{-1}) was used, and results were reported as gallic acid equivalents (GAE) per mg of tissue.

For the determination of oxidative stress from lipid peroxidation, a commercial kit (Cayman Chemical Company, Michigan, USA) was used. An extract was prepared from 0.15 g of plant tissue, 1.5 mL of phosphate buffer (100 mM, pH 7), and 3 mg of PVP. The homogenized sample was centrifuged (Select BioProducts, Select Spin 17R, USA) at 3,000 *x g* for 20 min at 4 °C. A standard curve was prepared from MDA (1:4). The reaction mixture consisted of 15 µL of the sample, 15 µL of SDS, and 600 µL of color reagent, which were placed in a water bath at 90 °C for 10 min, and the reaction was stopped in an ice bath. Samples were centrifuged at 3 000 *x g* for 20 min at 4 °C, and absorbance was quantified at 532 nm in a microplate reader (BioTek Instruments, Epoch, USA).

Data analysis

The experiments were established in a complete block design with 3 replications. All results were analyzed using analysis of variance (ANOVA), when it was needed a Tukey's *post hoc* test was performed for means comparison with $P \leq 0.05$. All statistical analyses were made using the NCSS software 1997. Graphs were generated using the SigmaPlot program 2008, student version.

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RESULTS

Phytochemical characterization of plant extracts by HPLC-ESI-MS

Table 1 shows the compounds identified by HPLC-ESI-MS in a bioherbicide extract composed of plant tissue from creosote bush (leaves), tarbush (leaves), pecan shells (solid waste), and eucalyptus (leaves) (56, 19, 12.5, and 12.5%, respectively). The analysis revealed 14 phytochemicals from different families, such as flavonols, flavones, hydroxycinnamic acids and catechins, among others. Among the key compounds were quercetin, a well-known flavonol celebrated for its antioxidant and bioactive properties, and (+)-gallocatechin, a catechin recognized for its significant antioxidant activity and various health benefits.

Table 1. Compounds identified by HPLC-ESI-MS from the extract of creosote bush, tarbush, pecan shells, and eucalyptus.

Retention time (min)	m/z ([M-H]⁻)	Identity	Family
4.188	316.7	6,8-Dihydroxykaempferol	Flavonols
4.807	316.8		
15.484	336.8	3-p-Coumaroylquinic acid	Hydroxycinnamic acids
16.197	284.8	Luteolin	Flavones
22.617	283.8	Geraldone	Methoxyflavones
24.552	283.7	Methylgalangin	Methoxyflavonols
27.747	608.7	Quercetin 3-O-xylosyl-glucuronide	Flavonols
29.107	304.8	(+)-Gallocatechin	Catechins
33.578	346.7	5-Heptadecylresorcinol	Alkylphenols
37.853	314.7	Rhamnetin	Methoxyflavonols
40.703	314.8	Isorhamnetin	Methoxyflavonols
45.421	298.9	4-Hydroxybenzoic acid 4-O-glucoside	Hydroxybenzoic acids
47.463	300.9	Quercetin	Flavonols
51.909	314.9	Protocatechuic acid 4-O-glucoside	Hydroxybenzoic acids
54.254	301.0	6-Hydroxyluteolin	Flavones

Germination and growth

The germination percentage occurred mainly during the first 4 days, which was modified by the effect of the treatments, where the low dose treatment of bioherbicidal extract increased germination in *C. foetidissima*, obtaining up to 68.52 ± 4.21%, compared to the control at 22.22 ± 5.24%, the medium dose at 35.19 ± 7.45%, and the high dose at 27.78 ± 6.55% treatments (Figure 2A). At 14 days after the application of the treatments, the germination percentage increased slightly for the control at 24.07 ± 5.90%, the low dose at 70.37 ± 4.00%, and the medium dose treatments at 37.04 ± 8.00%, except for the high dose treatment at 27.78 ± 6.55%, which no longer showed germination. These values were sustained until 45 days (Figure 2A). At 45 days after the application of the bioherbicidal extracts, the length of the seedling (aerial part) was measured. The low dose treatment showed the greatest growth of the aerial part at 12.54 ± 0.65 mm, compared to the control treatments at 5.41 ± 1.11 mm, medium dose at 7.74 ± 1.63 mm, and high dose at 5.62 ± 1.15 mm (Figure 2B). Dry biomass did not show significant differences between treatments, although there was a tendency towards lower dry biomass in the root of *C. foetidissima* seedlings

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under different treatments compared to the control. This trend was similar to that observed in the aerial part, where the seedlings of the control group showed higher dry biomass compared to the aerial part of the seedlings treated with the different bioherbicidal extracts (Figure 2C).

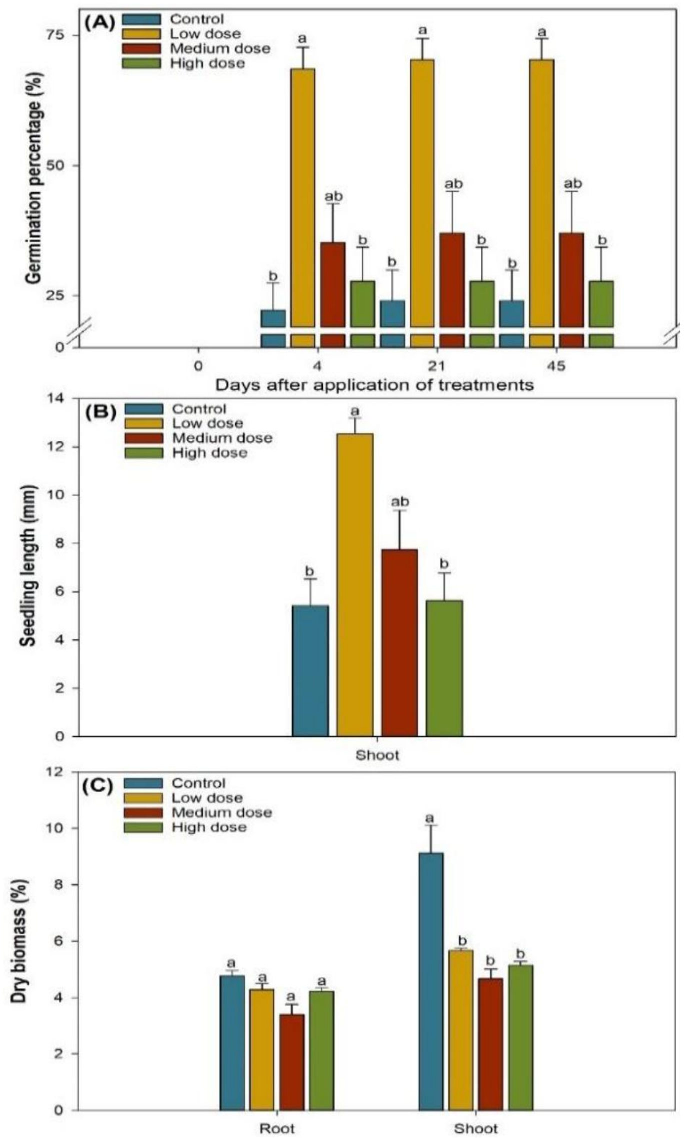

Figure 2. (A) Germination percentage, (B) seedling length, and (C) dry biomass percentage in root and aerial part of *Cucurbita foetidissima* with the application of different treatments based on bioherbicidal extracts (low, medium, and high doses). Bars indicate mean ± standard error. Different letters indicate significant differences by ANOVA and Tukey, $p \leq 0.05$.

Photosynthetic pigments and protein

The content of chlorophylls (a, b, and total) decreased in *C. foetidissima* seedlings in all those that were treated with the bioherbicidal extracts at different concentrations, compared to the control group (Figure 3A). Similarly, for carotenoid content, these pigments were found in lower

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concentration in *C. foetidissima* seedlings that were treated with the bioherbicidal extracts compared to the control group seedlings (Figure 3A). The total protein content did not show any modifications because of the treatments in both root and shoot of *C. foetidissima* seedlings (Figure 3B).

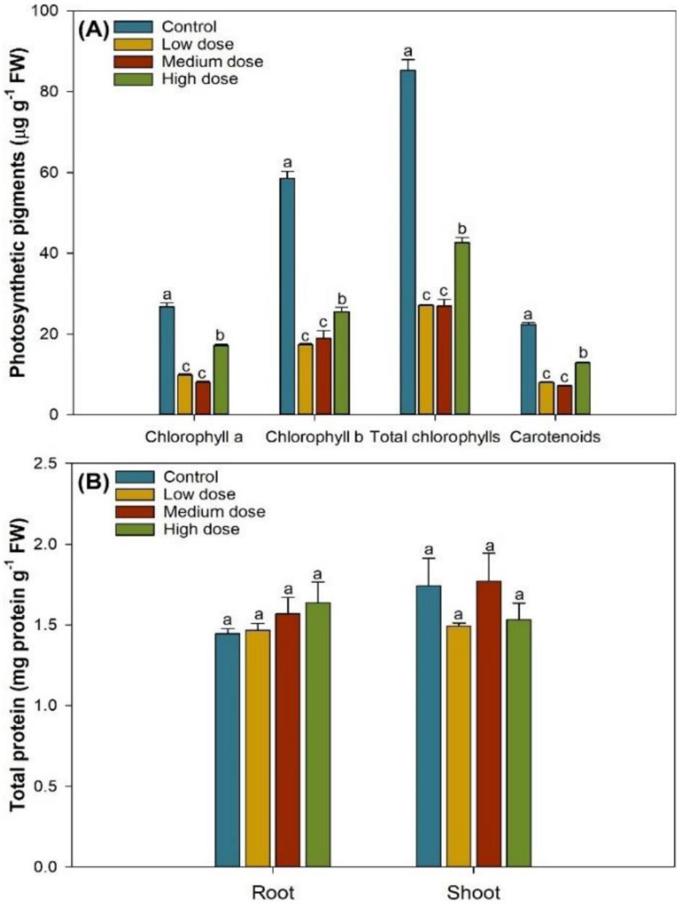

Figure 3. (A) Photosynthetic pigments (chlorophylls and carotenoids) in shoot and (B) total protein on seedling (root and shoot) of *Cucurbita foetidissima* with the application of different treatments based on bioherbicidal extracts (low, medium, and high doses). Bars indicate mean ± standard error. Different letters indicate significant differences by ANOVA and Tukey, $p \leq 0.05$.

Antioxidant enzymes

The specific activity of catalases was not altered by the treatments in the roots of *C. foetidissima* seedlings. However, in the shoots of *C. foetidissima* seedlings, the treatments of lower concentration of bioherbicidal extracts (low dose) decrease the enzymatic activity of catalases (Figure 4A). Peroxidase enzyme activity did not show changes in either root or shoot of *C. foetidissima* by the action of bioherbicidal extracts (Figure 4B).

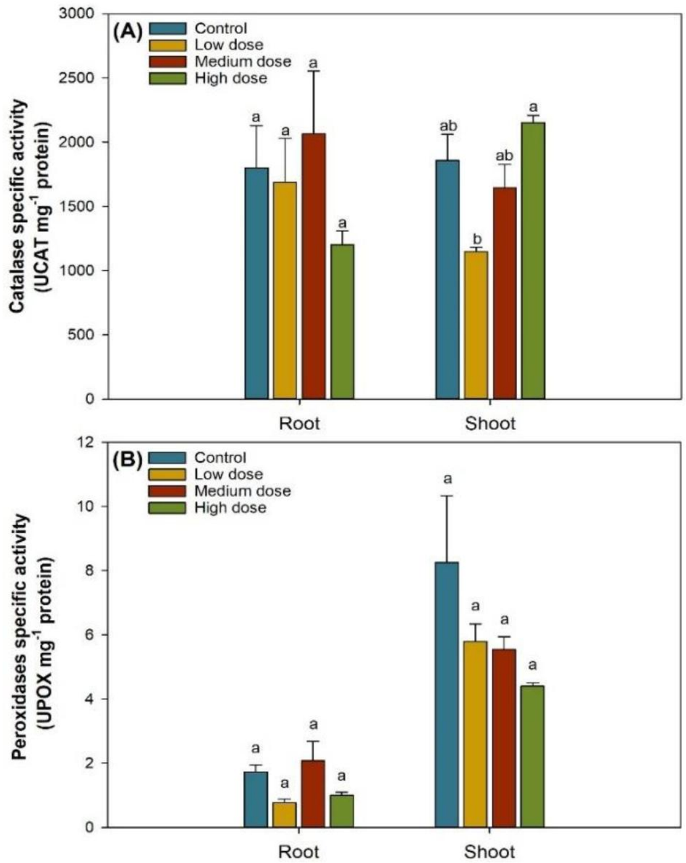

Figure 4. (A) Catalases (specific activity) and peroxidases (specific activity) on seedling (root and shoot) of *Cucurbita foetidissima* with the application of different treatments based on bioherbicidal extracts (low, medium, and high doses). Bars indicate mean ± standard error. Different letters indicate significant differences by ANOVA and Tukey, $p \leq 0.05$.

Antioxidants and oxidative stress

The content of phenolic compounds increased in both root and shoot of *C. foetidissima* seedlings in those treated with the bioherbicidal extracts compared to the control group seedlings (Figure 5A). Antioxidant capacity also increased by action of the treatments in both root and shoot, except for the medium dose treatment where a decrease in phenol content was also observed compared to the shoots of seedlings treated with the different concentrations of treatments (Figure 5B). This seems to be related to an increase in oxidative stress measured as lipid peroxidation, where the results show that only in the seedlings of the mentioned treatment (medium dose) oxidative stress increased compared to the rest of the tissues (root and shoot) and treatments evaluated (Figure 5C). In roots, no modification of oxidative stress was observed due to the action of the treatments (Figure 5C).

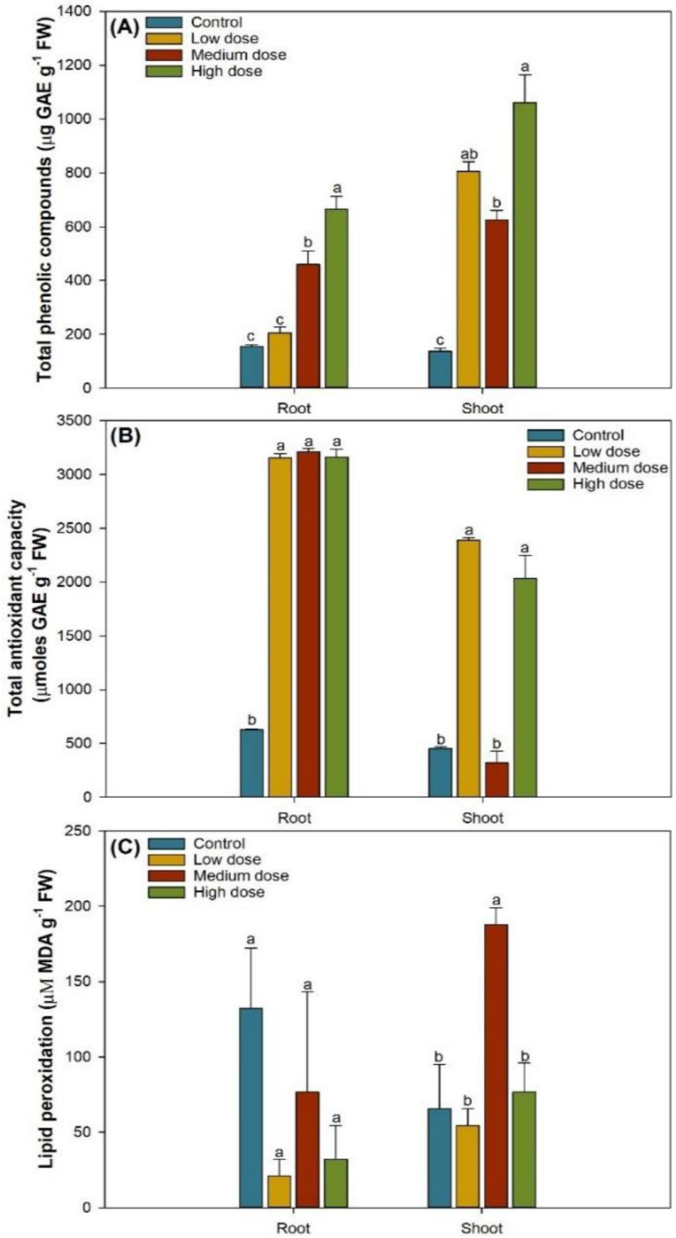

Figure 5. (A) Total phenolic compounds, (B) total antioxidant capacity, and (C) lipid peroxidation in root and shoot of *Cucurbita foetidissima* plantlets with the application of different treatments based on bioherbicidal extracts (low, medium, and high doses). Bars indicate mean ± standard error. Different letters indicate significant differences by ANOVA and Tukey, $p \leq 0.05$.

DISCUSION

Germination and growth

In this study, we observed that bioherbicidal extracts formulated primarily with creosote bush and tarbush, supplemented with pecan shell and eucalyptus leaf extracts, affected the germination of *C. foetidissima*. Specifically, germination increased at the lowest bioherbicide concentration but decreased progressively as the concentration increased. Similar findings were reported by Khan *et al*. (2012), who showed that extracts of *Rumex crispus* inhibited germination in wheat (*Triticum aestivum* L., cv. Atta Habib) and sunflower (*Helianthus annuus* L.) seeds by up to 70 and 80%, respectively, while maize (*Zea mays* L., cv. Azam) demonstrated high tolerance, suggesting species-specific responses to bioherbicidal effects. For *C. foetidissima*, the observed reduction in germination is likely due to the concentration-dependent effects of allelopathic compounds. The impact of these compounds varies widely depending on the species from which they are derived, the concentration applied, the specific plant part used for extraction, and the target species (Gulzar *et al.* 2014, Nadeem *et al.* 2023, Motmainna *et al.* 2023, Shadab *et al.* 2024). Additionally, *C. foetidissima* may alter its reproductive strategies in response to exposure to allelochemicals from other plants, such as *Brassica nigra* (Rodríguez *et al.* 2016), which could explain why germination was not inhibited at low doses of *L. tridentata*/*F. cernua* extracts. Phenolic compounds in these extracts have been shown to delay seed germination by inhibiting amylase activity (Radhakrishnan *et al.* 2018). It has been reported that *F. cernua* contains caftaric acid, vicenin-2, and a variety of flavonoids that inhibit alpha-amylase and alpha-glucosidase (Aranda-Ledesma *et al.* 2022). Additionally, Mata *et al*. (2003) identified three phytotoxins in *F. cernua* (dehydroflourensic acid, flourensiol, and methyl orsellinate), attributed with allelopathic properties. However, these compounds were not identified in the formulation used, but other phenolic compounds were detected, which could contribute to the reduced germination of *C. foetidissima* at higher extract concentrations, as observed. The allelopathic effects of *F. cernua* are similar to those of *L. tridentata*, as they may facilitate their own proliferation at the expense of reducing the proliferation of other plants in grassland ecosystems (DeLoach *et al.* 1986). In *L. tridentata*, a variety of polyphenols have been identified, including nordihydroguaiaretic acid, ellagic acid, gallic acid, tannins, catechins, and quercetin, which can translocate from the aerial parts to the roots and exert allelopathic effects in the soil, although these compounds tend to degrade rapidly (Hyder *et al.* 2002). In the extracts of the formulation used, a higher quantity of compounds related to flavonols, flavones, catechins, and quercetin was detected. Therefore, these phenolic compounds are likely to contribute significantly to the bioherbicidal effect.

The negative effect of bioherbicidal extracts on *C. foetidissima* shoot growth observed in the present study was dose-dependent, which agrees with the results reported by Gulzar *et al*. (2014). Likewise, they agree with that reported by Duarte *et al*. (2023) who used extracts of *Acacia dealbata* and *Oxalis pes-caprae* on different weeds and appreciated a decrease in growth that depends on the concentration of the extracts and the weed species. Meanwhile, Rodríguez *et al*. (2016) reported that *C. foetidissima* decreased its growth when exposed to allelochemicals of *B. nigra*, which confirms its susceptibility to allelopathic compounds. Wazir *et al*. (2011) suggest that the use of bioherbicides from plant extracts is an effective and sustainable alternative to the use of synthetic chemical herbicides for weed control. Where even, weeds themselves are a rich source of allelopathic compounds (Sidhu *et al.* 2023), which can affect economically important crops (Nadeem *et al.* 2023). Sidhu *et al*. (2023) reported that extracts of three weeds (*Amaranthus viridis*, *Achyranthes aspera* and *Lantana camara*) negatively affected rice germination and growth. The evaluation of these last variables is very necessary to determine the herbicidal potential of natural extracts. Soto-Maldonado *et al*. (2022) evaluated walnut shells (solid waste) extract obtained from microwave and ultrasound, which they tested on broadleaf and narrowleaf weed plants. The results showed phytotoxic effects of walnut shells extracts, mainly on broadleaf plants. This effect suggests a potential use of the walnut shells extract as a bioherbicide. Therefore, the addition of walnut shells extract to formulations could enhance the bioherbicidal effect and lead to reduced growth of *Cucurbita* plants, which are characterized by their broad leaves.

The dry matter of both roots and shoots of *C. foetidissima* decreased under the influence of bioherbicidal extracts. Allelopathic compounds from *F. cernua* have been reported to inhibit radicle growth in *Amaranthus hypochondriacus* and *Echinochloa crus-galli* (Mata *et al*. 2003), which could similarly reduce root dry matter. Additionally, biomass of other plant parts may decrease in the presence of allelopathic compounds (Gulzar *et al.* 2014, Shadab *et al.* 2024). Since plant dry weight reflects the synthesis and accumulation of organic compounds (Erida *et al.* 2019), the observed reduction in *C. foetidissima* suggests interference with compound synthesis. Kanatas (2020) highlighted the allelopathic effects of *Eucalyptus globulus* and *E. camaldulensis*, whose phenolic compounds and terpenes inhibit the growth and germination of various weeds in Mediterranean regions. *E. globulus* showed herbicidal efficacy against weeds such as *Cyperus rotundus* and *Amaranthus retroflexus*, while *E. camaldulensis* reduced the dry weight and germination of weeds like *Bromus rigidus* and *Avena fatua*. Incorporating eucalyptus extracts into the formulation could thus enhance dry matter reduction in *C. foetidissima*.

Photosynthetic pigments and protein

Bioherbicidal extracts also decreased photosynthetic pigments (chlorophylls and carotenoids) in *C. foetidissima* shoots. High concentrations of *Lepidium didymum* extracts have been shown to reduce chlorophyll and carotenoid content in *Lens culinaris* and *Morus alba* by over 40% (Shadab *et al.* 2024), along with a decrease in protein levels as extract doses increase (Gulzar *et al.* 2014). These effects are attributed to phenolic compounds that disrupt plant functions, including photosynthesis (Kostina-Bednarz *et al.* 2023). The synthesis rate of pigments such as chlorophylls and carotenoids can be inhibited by plant extracts with herbicidal potential, depending on the extract source and plant tolerance (Radhakrishnan *et al.* 2018). Motmainna *et al*. (2023) further noted that allelochemicals can alter cellular structures and modulate key enzymes. The primary mode of action of bioherbicidal extracts observed in this study is the reduction of photosynthetic pigment content, potentially impairing photosynthesis in *C. foetidissima*.

The protein content was not modified in any of the tissues of *C. foetidissima* plants by the effect of bioherbicidal extracts, perhaps due to a buffering effect of *C. foetidissima* to stress by the presence of allelopathic compounds. Several authors have indicated that phenolic compounds in plant extracts used for their allelopathic properties influence protein content. Erida *et al*. (2019) report that gallic acid from *A. conyzoides* can inhibit the growth of other plants by inducing protein denaturation. While Gulzar *et al*. (2014) reported that phenolic compounds reduce the incorporation of certain amino acids into proteins. Likewise, Radhakrishnan *et al*. (2018) indicated that plant extracts with allelopathic properties modify and negatively affect protein metabolism, including chlorophyll-binding proteins, which decreases their content.

Antioxidant enzymes

Catalase activity in *C. foetidissima* shoots was only negatively affected when a low dose of bioherbicidal extracts was used, whereas peroxidases showed no changes by action of the treatments in any of the tested tissues of *C. foetidissima*. Hasan *et al*. (2021) indicated that catalases are antioxidant enzymes that regulate the presence of reactive oxygen species (ROS), so they are important in the regulation of plant cell detoxification, whose activity was decreased along with POD enzyme in rice plants that were exposed to allelochemical compounds, which may increase lipid peroxidation. In general, CAT and POD enzymes are known to detoxify H_2O_2 by degrading it into water (H_2O) and oxygen (O_2). Allelochemical compounds increase the content of free radicals and H_2O_2, resulting in irregular activities of CAT and POD enzymes (Radhakrishnan *et al.* 2018). However, *Cucurbita* species have high amounts of phytochemicals with antioxidant activity (Salehi *et al.* 2021). Particularly, *C. foetidissima* presents high concentrations of catechol (0.8 and 1.9 g 100 g^{-1} DW) in all its tissues (Martinez *et al.* 2021). The antioxidant compounds of *C. foetidissima* could help to reduce the ROS content in this plant and the activity of antioxidant enzymes may not present a major modification.

Antioxidants and oxidative stress

The content of phenolic compounds and antioxidant capacity increased in both root and shoot of *C. foetidissima* treated with bioherbicidal extracts. Radhakrishnan *et al*. (2018) mention that the antioxidant capacity can be modified by ROS production caused by the extracts with allelopathic compounds. Pinto *et al*. (2023) evaluated extracts from fresh and dried eucalyptus leaves, which resulted in a decrease in the germination of *Portulaca oleracea*. They also observed a reduction in biomass and chlorosis in the plants, which they attributed to an increase in ROS production. Therefore, all the allelopathic compounds in the formulated product, including the eucalyptus extracts, may be affecting *C. foetidissima* and its antioxidant content as a mechanism to counteract the damage caused by increased ROS. The highest oxidative damage evaluated as lipid peroxidation was observed in *C. foetidissima* shoots treated with the mean dose of bioherbicidal extracts, which coincides with a decrease in the content of phenolic compounds and antioxidant capacity. In this regard, Bordin *et al*. (2021) and Hasan *et al*. (2021) mention that oxidative stress is increased in plants due to exposure to bioherbicides and their components. Other natural extracts used as bioherbicides have generated excessive ROS production, causing an imbalance in the antioxidant system and damage to the integrity of cell membranes, leading to an increase in MDA content or lipid peroxidation (Li *et al.* 2023). This damage indicates toxicity of plant extracts that trigger damage at different levels in plant cells resulting in decreased plant growth or inhibition of germination (Radhakrishnan *et al.* 2018). The effects of bioactive compounds depend on various factors, such as: biotic and abiotic environmental conditions, soil, study method and model, presence of organic matter, among others (Choudhary *et al.* 2023, Duarte *et al.* 2023). Therefore, the antioxidant mechanism can be influenced by various factors, in this case, the study model on a substrate can modify the effects on *C. foetidissima*, so further studies are required to complete the understanding of the mechanisms of bioherbicidal action.

CONCLUSIONS

The bioherbicidal extracts are significant for their pre-emergent effect on *C. foetidissima*, with a greater impact observed at higher concentrations. The mechanism of action of the allelopathic compounds involves a reduction in dry biomass and photosynthetic pigments (chlorophylls and carotenoids), modification of catalase enzyme activity, and an increase in total phenolic content and antioxidant capacity in *C. foetidissima* shoots. Further evaluation of the potential post-emergent effects of these extracts and their application as bioherbicides on other weeds is necessary.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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