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Antioxidant and antibacterial activity of aqueous, ethanolic and acetonic extracts of *Pimenta dioica* L. leaves

[1]Sánchez-Zarate, A., [2]Hernández-Gallegos, M. A., [1]Carrera-Lanestosa, A., [3]López-Martínez, S., [1]Chay-Canul, A. J., [4]Esparza-Rivera, J. R. and [1]*Velázquez-Martínez, J. R.

[1]Biotic Products Development Laboratory, Academic Division of Agricultural Sciences, Juarez Autonomous University of Tabasco, Villahermosa, Tabasco, 86280, México
[2]Academic Division of Multidisciplinary of Jalpa de Mendez, Juarez Autonomous University of Tabasco, Jalpa de Méndez, Tabasco, 86205, México
[3]Academic Division of Biological Sciences, Juarez Autonomous University of Tabasco, Villahermosa, Tabasco, 86039, México
[4]Faculty of Chemical Sciences, Gómez Palacio Unit, Juarez University of the State of Durango, Gómez Palacio, Durango, 35010, México

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Abstract

The aim of the present work was to evaluate the antioxidant and antibacterial activity of crude extracts (aqueous, ethanolic, and acetonic) of *Pimenta dioica* L. leaves collected in Tabasco, México. The following tests were carried out: total phenols (Folin-Ciocalteu assay), antioxidant activity (DPPH, ABTS, and FRAP methods), and antibacterial activity (disc diffusion and minimum inhibitory concentration techniques) using Gram-positive and Gram-negative bacteria. To identify compounds in the extracts, UHPLC-QToF was utilised. The acetonic extract (AE) yielded the highest phenolic content (626.29 ± 28.11 mg GAE/g dry extract, $p < 0.05$). The phenolic content of the aqueous (WE) and ethanolic extracts (EE) (488.29 ± 15.56 and 516.35 ± 4.96 mg GAE/g dry extract, respectively) did not show a significant difference. Likewise, AE yielded the highest antioxidant activity of 52.26 ± 11.74 µg/mL (IC_{50}), 116.90 ± 10.82 µg/mL (IC_{50}), and 7.52 ± 0.16 mM TE/g dry extract for DPPH, ABTS, and FRAP assay, respectively. Again, the EE and WE did not show a statistical difference for the DPPH and ABTS assays, while the antioxidant activity with the FRAP assay showed statistically different results for the three extracts in the order of AE > EE > WE. In addition, all three extracts showed antibacterial activity, with *B. cereus* and *S. aureus* (Gram-positive bacteria) more sensitive than *S. Typhimurium* and *E. coli* (Gram-negative bacteria); however, the minimum inhibitory concentrations were high with respect to the positive control (amikacin). Using the HPLC analysis, it was possible to detect and confirm the presence of some compounds such as phenols, flavonols, triterpenes, sapogenins, and polyalcohol in AE, EE, and WE . The antioxidant activity was closely related to the content of total phenols, although not all phenolic compounds had antibacterial activity. Extracts of *P. dioica* leaves are a source of phenolic compounds and can be used as antioxidant agents in the food, cosmetic, and pharmaceutical industries.

Introduction

Pimenta dioica L. is a plant belonging to the Myrtaceae family and commonly known as Jamaica pepper, thick pepper, Tabasco pepper, and allspice. Allspice was so named because the flavour of the dried berry is reminiscent of a blend of spices such as clove, nutmeg, and cinnamon (Lim, 2012). This evergreen tree, which is medium in size, is native to the West Indies (Antilles) and Central America, and is most abundant in Jamaica (Padmakumari *et al.,* 2011). In Mexico, it is widely distributed in the tropical regions of Veracruz, Chiapas, Tabasco, Campeche, and Yucatan. Traditionally, the berries are harvested when they are not completely mature, and afterwards, dried in the sun to obtain their characteristic brown colour (Lim, 2012).

The dried berries and leaves of *P. dioica* are mainly used as spice in meat, fish, and bakery products, as well as to improve the flavour in desserts, pickles, ketchup, soups, and sauces. In early Mesoamerican civilisations, it was used as a flavouring in traditional beverages made out of corn and cacao. The cosmetic industry adds the essential oil of this pepper to soap

*Corresponding author.
Email: jrodolfovelazquez@gmail.com

and perfume formulations, while the pharmaceutical industry uses it for improving the flavour of medicinal products (Lim, 2012). Besides, *P. dioica* has been regularly used in alternative medicine to alleviate stomach illnesses such as diarrhoea and gas, and has also been used as an appetite stimulant. It has also been used as a treatment agent for rheumatism, arthritis, chills, coughing, bronchitis, and neuralgia (Charles, 2013).

Crude extracts, essential oils, and purified fractions of *P. dioica* leaves and berries have shown biological activities such as anti-hypertensive, anti-inflammatory, analgesic, anti-pyretic, anti-ulcerous, anti-cancerogenic, anti-diabetic, nematicide, acaricide, anti-poisonous, antioxidant, and antimicrobial agents (Al-Rehaily *et al.,* 2002). These properties are attributed to several compounds contained in the *P. dioica* berries and leaves, including eugenol and methyl eugenol (phenolic derivatives), and beta-caryophyllene (terpene) (Vadlapudi and Kaladhar, 2012).

However, despite the existing information regarding the beneficial biological activities of the leaves of *P. dioica*, they are only used as an alternative medicinal agent, as well as a cuisine ingredient. In Mexico, *P. dioica* leaves are discarded after collection and post-harvest processing thus considered agro-industrial waste (Martínez *et al.,* 2013). These leaves represent cheap raw materials for obtaining phytochemicals.

Chemical compounds that show antioxidant activity include vitamins, carotenoids, and phenolic compounds. Water, ethanol, and acetone can extract considerable amounts of phenolic compounds. However, the content of phenolic compounds extracted can vary depending on the plants and affinity for the solvents. All phenolic compounds have antioxidant activity, but only some have antimicrobial activity (Tiwari *et al.,* 2011). In addition to the importance of phytochemicals with antioxidant activity such as drugs, there is considerable interest in the food industry to find plant antioxidants to replace synthetic ones, being that, they are assumed safe if they come from plants for food use (Chanwitheesuk *et al.,* 2005). Furthermore, there are no reports regarding the antioxidant and antibacterial activities of *P. dioica* from Mexico. Hence, the aim of the present work was to evaluate the phenolic content, antioxidant, and antimicrobial activities of extracts of the leaves of *P. dioica* collected in Tabasco, Mexico.

Materials and methods

Bacterial strains

The bacterial strains used were *Staphylococcus aureus* ATCC 25923, *Salmonella* Typhimurium ATCC 14028, *Escherichia coli* ATCC 25922, and *Bacillus cereus* ATCC 11778; and they were donated by the Food and Biotechnology Department, Faculty of Chemistry, National Autonomous University, México.

Vegetative material

Pimenta dioica L. leaves were collected from a culture during the harvest of the berries in October of 2017 in Jalapa, Tabasco, México (17° 49’18.2 "N 92°47'44.6"W). The leaves were washed using tap water, and excess moisture was removed using a manual centrifuge. The leaves were then dehydrated at room temperature for 5 d in a room equipped with an environmental dehumidifier (LG®). The dried samples were ground in a mechanical mill and sieved using a no. 60 mesh. The obtained powder (1% humidity) was stored at 4°C, and protected from light and moisture until further extraction.

Extraction

The extracts were obtained by mixing 5% of the grounded sample with each of the three different extraction solvents: deionised water (aqueous extract, WE), ethanol:deionised water (70:30 v/v, ethanolic extract, EE), and acetone:deionised water (70:30 v/v, acetonic extract, AE). The WE were prepared by heating at 90°C for 10 min in a water bath with periodic agitation. The heated mixture was filtered using Whatman No. 1 filter paper and microfiltered with Millipore® equipment using a nitrocellulose membrane with a 0.45 µm pore diameter. The EE and AE extracts were obtained by macerating with orbital agitation at 150 rpm for 48 and 24 h, respectively. The EE was filtered and microfiltered following the same procedure used for WE preparation. Meanwhile, the AE was centrifuged at 4,000 *g*, and the recovered supernatant was used as the final extract. Extracts were placed in dark containers, and stored at 4°C.

The water in the WE were eliminated by lyophilisation. The ethanol and acetone were eliminated using a rotary evaporator (45 and 50°C, respectively), and the rest of the water for EE and EA was eliminated in a vacuum stove at 60°C. The dried extracts were ground, and stored at 4°C, protected from light and moisture until further analyses.

Determination of total phenolic content

Total phenolic content (TPC) was determined following a spectrophotometric method using the Folin-Ciocalteu reagent (Singleton *et al.,* 1999) with modifications. Briefly, 125 µL of extract were mixed with 625 µL of diluted Folin-Ciocalteu reagent with deionised water (1:10) and 500 µL of 7.5% sodium

carbonate solution. The mixture was allowed to react for 45 min in darkness. The absorbance was read at a wavelength of 760 nm. The TPC was calculated using a standard curve prepared with gallic acid as a reference, and the results were reported as mg of gallic acid equivalent per g of dry extract (mg GAE/g dry extract). All measurements were replicated three times.

Antioxidant activity determination

DPPH• radical scavenging activity assay

The DPPH• free radical scavenging ability in the extracts was evaluated following the method proposed by Brand-Williams *et al.* (1995) with modifications. Briefly, 200 µL of the extract was mixed with 2 mL of DPPH• solution (0.125 mM, in 80% methanol) and allowed to react in darkness for 60 min at room temperature. The antioxidant capacity was evaluated by measuring the mixture absorbance at 520 nm. The IC_{50} (concentration of extract required to inhibit 50% of radical, µg/mL) values were calculated by linear regression analysis. The radical-scavenging percentage was calculated using Eq. 1:

$$\% \text{ inhibition} = (Abs\ control - Abs\ sample)/(Abs\ control) \times 100 \quad \text{(Eq. 1)}$$

where, *Abs control* = absorbance of the mixture of reagents without adding either sample or standard; and *Abs sample* = absorbance of the mixture added with either sample or standard reagent.

ABTS•+ radical scavenging activity assay

The free radical-scavenging activity was determined following the ABTS•+ radical cation decolorisation assay (Re *et al.,* 1999) with modifications. The ABTS•+ radical solution was prepared with 2.45 mM potassium persulfate and 7 mM ABTS•+ (1:1), and allowing it to react for 12 - 16 h at room temperature protected from light. The ABTS•+ solution absorbance was adjusted with methanol to obtain an absorbance of 0.700 ± 0.001 at 734 nm. The radical scavenging activity was determined by mixing 10 µL of the extract with 990 µL of the adjusted solution, and allowing to react for 6 min. The absorbance mixture was read at 734 nm. The results were reported as IC_{50} (µg/mL), which was calculated following the same procedure as in DPPH assay.

Reducing power of ferric ion FRAP (Ferric reducing antioxidant power)

This method was used as an antioxidant activity indicator based on the reducing power of a sample (Benzie and Strain, 1999) with modifications. The FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ in the proportion of 10:1:1 at room temperature. Next, 1,200 µL of fresh FRAP reagent was mixed with 40 µL of extract and 120 µL of distilled water. The mixture absorbance was measured at 593 nm after 30 min of the reaction at room temperature. A standard curve was prepared using Trolox as a reference standard, and the results were reported as mM Trolox equivalent per g dry extract (mM TE/g dry extract). All solutions used in the assay were prepared on the same analysis day.

Antibacterial activity

Disc diffusion

The antibacterial activity was evaluated using the disc diffusion (DD) technique (Abbes *et al.,* 2014) against *S. aureus, S.* Typhimurium, *E. coli*, and *B. cereus* which were activated in brain heart infusion (BHI) broth from aliquots in glycerol stored at -20°C. Petri dishes were inoculated with 0.1 mL of each bacterial culture at a concentration of 10^8 CFU/mL. Four 6 mm diameter Whatman No. 1 paper discs were placed on each Petri dish. Two discs were diffused with 10 mg of extract, one disc with 30 µg of antibiotic (amikacin) as a positive control (Manandhar *et al.,* 2019), and one with the corresponding solvent of each extract as a negative control. The Petri dishes were incubated at 37°C for 12 - 24 h, following which the zones of inhibition diameter (in mm) were recorded.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined using the microplate dilution technique. *S. aureus, S.* Typhimurium, *E. coli,* and *B. cereus* were activated in Mueller Hinton (MH) broth. 96-well sterile microplates of 500 µL capacity were used for the assay. Each extract was applied at a serial dilution rate (1:1) from 50 to 0.39 mg/mL. The WE were diluted using sterile distilled water. The EE and AE were diluted using sterile DMSO solution (10%). To each well were added 100 µL of MH broth inoculated with the respective bacterial strain and 100 µL of either diluted extract or control (positive or negative). Amikacin and chloramphenicol were used as positive controls (Manandhar *et al.,* 2019) at a dilution rate from 2 to 0.015 and 4 to 0.03 mg/mL, respectively. The negative control was sterile with distilled water and 10% DMSO from 10 to 0.0075%. Each microplate was agitated for 5 min at 150 rpm in a vortex shaker (IKA™). Microplates were incubated for 24 h at 37°C. Bacterial growth was assessed using 100 µL to 1% of 3-(4,5-dimetiltiazol-2-ilo)-2,5-difeniltetrazol) bromure (MTT) as a viability indicator, which changed

the solution colour from yellow to violet after 1 h. The concentration of the first well that did not change colour was considered the MIC. 10 µL from the well with the MIC was taken and inoculated in a Petri dish containing MH agar and incubated for 24 h. The results were reported as the bacteriostatic activity if there was bacterial growth, and bactericidal activity if there was no bacterial growth (Balouiri *et al.,* 2016).

HPLC analysis

The analysis of the compounds in the extracts was carried out using UHPLC-QToF. Briefly, 1 g dried extract was dissolved in 1.5 mL MeOH. The mixture was subsequently sonicated for 10 min, and subjected to solid-phase extraction with a C_{18} column using methanol as eluent. The solvent was removed at 40°C under reduced pressure on a rotary evaporator. Next, 1 mL of each extract was prepared at a concentration of 100 µg/mL. The solutions were filtered through a 0.2 µm nylon filter, and formic acid was added to obtain a final concentration of 0.1%. Solutions were sonicated for 5 min.

A Waters® Acquity UPLC® Class I system coupled to a Xevo® G2 XS QToF mass spectrometer equipped with an electrospray ionisation source (Waters Corp., Milford, MA, USA) was used. An Acquity UPLC HSS T3 C_{18} 2.1 × 100 mm column and particle size 1.8 µm was used at 40°C with a duration of 35 min. Mobile phase A: water:acetic acid (99.9:0.1, v/v), phase B: acetonitrile (100%); linear elution gradient: 97% A and 3% B (0 - 30 min), 3% A and 97% B (30 - 32 min), 97% A and 3% B (32 - 35 min); flow rate: 0.2 µL/min, and injection volume: 5 µL of the sample at 7°C.

The mass spectrometer was operated in the negative ionisation mode (ESI-) and sensitivity mode, in the mass range set at 100 to 1,200 m/z. It was used as a reference standard leucine/enkephalin, [MH]- = 554.2615. For the acquisition and processing of data, Masslynx version 4.1 software was used.

Statistical analysis

Each test was performed in triplicate, and the results were expressed as mean ± standard deviation. The results were analysed using a one-way analysis of variance (ANOVA), and the means were compared using the Tukey ($p < 0.05$) multiple range test. Pearson's correlation coefficients ($p < 0.05$) were calculated between the antioxidant activity values and the total phenolic content values of the extracts. SAS software version 9.0 was used for statistical analysis.

Results and discussion

Extraction yield and total phenolic content

The aim of the extraction process is to obtain the highest yield of compounds with high quality. Bioactivity and antioxidant activity in an extract depends on several factors such as solvent polarity, pH, extraction temperature and time, and sample composition (Gullón *et al.,* 2017). Extraction yields (Table 1) were from 28.3 to 33.3%, with the highest yield from AE ($p < 0.05$). This proved that the extraction yield depended on the solvent polarity, which agrees with the results reported in other studies performed with Myrtaceae family plants, like *Eucalyptus globulus* leaves (Rodrigues *et al.,* 2018) and *Eugenia caryophyllus* flowers (Hemalatha *et al.,* 2016). AE also showed the highest TPC of all extracts (626.29 ± 28.11 mg GAE/g dry extract). There was no significant difference ($p > 0.05$) between WE and EE. Several studies have stated that acetone-water extracts increase the solubility of the phenolic compound, thereby obtaining high TPC in such extracts (Do *et al.,* 2014; Złotek *et al.,* 2016). However, selecting an adequate solution for the most efficient extraction will depend on the used material, as well as on the further extract used (Hidalgo and Almajano, 2017; Granato *et al.,* 2018). Regarding *P. dioica* studies, there was limited information because the existing studies are only based on the essential oil extraction. On the other hand, the TPC of all the extracts evaluated in the present work was higher than the pepper powder ethanolic extract obtained in 50% ethanol as reported by Dearlove *et al.* (2008) (122.1 ± 7.0 mg GAE/g dry extract), and that obtained with the Soxhlet equipment (102.1 ± 1.80 mg GAE/g dry extract) reported by Nayak *et al.* (2008), which was 80% lower than the TPC obtained in the present work. In another study published by Dharmadasa *et al.* (2015) using mature and immature leaves, the TPC (94.15 ± 2.14 to 99.09 ± 3.65 mg GAE/g dry extract) was lower than that obtained in the present. Therefore, it is important to emphasise that even with the low values of TPC (488.29 ± 15.56 mg GAE/g dry

Table 1. Extraction yield and total phenolic content of *P. dioica* leaves crude extracts.

Extract	Extraction yield (%)	Total phenolic content (mg GAE/g dry extract)
Aqueous	28.3 ± 1.02[b]	488.29 ± 15.56[b]
Ethanolic	32.3 ± 0.96[a]	516.35 ± 4.96[b]
Acetonic	33.3 ± 0.73[a]	626.29 ± 28.11[a]

Values are means ± standard deviations of triplicates (n = 3). Means in the same column followed by a different superscript lowercase letter are significantly different ($p < 0.05$).

extract) obtained in AE, the value was around five times higher than those reported by other researchers who used organic solvents. AE is also an important option for extraction because of its harmless nature.

Antioxidant activity

DPPH• radical scavenging activity

The results of the DPPH radical scavenging activity (Table 2) showed that the extraction solvent significantly affected the radical scavenging of *P. dioica* extracts. AE was the most active with the lowest values of IC_{50} (52.26 ± 11.74 µg/mL). On the other hand, there was no significant difference between WE and EE. However, in other studies using common south Asian plants, the best values of IC_{50} were obtained in the ethanolic extracts (Do *et al.,* 2014; Dhanani *et al.,* 2017). The difference in the efficiency to capture the DPPH radical between the extracts was mainly due to the existence of phenolic and polyphenolic compounds with different chemical characteristics and polarities, based on the characteristics of the solvent used (Yakoub *et al.,* 2018).

Table 2. Antioxidant activity (DPPH, ABTS, and FRAP assays) of *P. dioica* leaves crude extracts.

Extract	DPPH (µg/mL, IC_{50})	ABTS (µg/mL, IC_{50})	FRAP (mM TE/g dry extract)
Aqueous	74.51 ± 5.45^{b}	141.73 ± 1.49^{b}	6.36 ± 0.11^{c}
Ethanolic	71.31 ± 9.24^{b}	123.79 ± 16.36^{ab}	6.76 ± 0.18^{b}
Acetonic	52.26 ± 11.74^{a}	116.90 ± 10.82^{a}	7.52 ± 0.16^{a}

Values are means ± standard deviations of triplicates (n = 3). Means in the same column followed by a different superscript lowercase letter are significantly different ($p < 0.05$).

ABTS•+ radical scavenging activity

Table 2 shows the results of the ABTS•+ radical assay. AE and EE were the most active and showed significant difference between each other. Therefore, we can state that the evaluated extracts were affected by the solvent polarity, which resulted in a determined capacity by the solubilised phenolic compounds (Hayouni *et al.,* 2007). To the best of our knowledge, there have been no other studies reporting the ABTS•+ radical scavenging capacity of *P. dioica* leaves extracts using water, acetone, and ethanol as documented in the present work.

Reducing power of the ferric ion FRAP

The results of the FRAP assay (Table 2) show significant differences among the antioxidant activity of all extracts ($p < 0.05$). AE showed the highest antioxidant capacity, followed by EE and WE. The FRAP assay evaluates the reducing power of the extracts. Regularly, the reducing properties are associated with the compounds contained in a sample capable of donating hydrogen atoms in order to break the free radical chains (Loizzo *et al.,* 2016). The antioxidant activity results from the FRAP assays of all extracts were higher than 6 mM TE/g dry extract, which are higher than those results published by Dharmadasa *et al.* (2015) using a methanolic extract of *P. dioica* leaves collected in Sri Lanka. The extracts were sorted in the following order: AE > EE > WE. The antioxidant activity of the extracts in the present work is also higher than that reported by other studies (Kim *et al.,* 2013; Fernandes *et al.,* 2016; Zahin *et al.,* 2016).

Correlation between antioxidant activity and total phenolic content

The Pearson correlation coefficients between the antioxidant activity assays and the TPC determined by the Folin-Ciocalteu method showed a significant correlation (Table 3). However, the correlation coefficients were moderate between the TPC and the ABTS assays (r = -0.6949), and high for the DPPH and FRAP assays (r = -0.8288 and r = 0.9127, respectively). The negative values of the correlation coefficient indicate that the lower the IC_{50} values, the greater the antioxidant activity. Antioxidants scavenge free radicals by three mechanisms:

Table 3. Correlation between antioxidant activity and total phenolic content of *P. dioica* leaves extracts

Assay (expressed in units)	*Total phenolic content vs antioxidant activity		
	Equation	Pearson´s coefficient (r)	Significance
DPPH (IC_{50})	$y = 147.159 - 0.149235x$	−0.828837	$p < 0.01$ ($p = 0.0058$)
ABTS (IC_{50})	$y = 213.388 - 0.158035x$	−0.694934	$p < 0.05$ ($p = 0.0377$)
FRAP (mM TE/g)	$y = 2.892 + 0.007336x$	0.91267	$p < 0.01$ ($p = 0.0006$)

* = Results expressed in mg of GAE/g dry extract.

hydrogen atom transfer, electron transfer, and a combination of both mechanisms. The ABTS, FRAP, and DPPH assays are based on the electron transfer mechanism. However, these methods differ among each other in relation to the principles of the chemical reaction, the target molecule, and the way they express the results (Oliveira *et al.,* 2015). The moderate correlation values between the ABTS assay and TPC (r = -0.6949) could be attributed to other compounds that were not determined by the Folin-Ciocalteu method, and that interacted with the radical ABTS•+ and had antioxidant capacities. One drawback related to this method is that the ABTS•+ radical cation is reactive towards most antioxidants, not only the phenolic compounds present in the extracts (Martín-Gómez *et al.,* 2020). On the other hand, a high correlation between the antioxidant activity and TPC proved that the phenolic compounds exerted most of the antioxidant capacity of the extract (Fernandes *et al.,* 2016). Similar results in plant extracts were reported by Carvalho *et al.* (2015) and Nascimento *et al.* (2014). In the case of *P. dioica* berries, Turgay and Esen (2015) also reported a high correlation (r = 0.81) between the DPPH assay and TPC. The antioxidant activity of plant extracts was dependent not only on the vegetative material but also on the interaction of the phenolic compounds contained in the sample, as well as their structure and concentration (Hayouni *et al.,* 2007; Craft *et al.,* 2012).

Antibacterial activity

Discs diffusion

All three extracts evaluated showed antibacterial activity according to the disc diffusion method (Table 4). Gram-positive bacteria (*B. cereus* and *S. aureus*) were the most sensitive to *P. dioica* extracts with inhibition zones of 8.25 to 15.25 mm, with WE showing the highest antibacterial activity. Gram-negative bacteria (*S. Typhimurium* and *E. coli*) were less sensitive to the application of the extract with inhibition zones of 7.5 to 9.25 mm, and there was no significant difference among *P. dioica* extracts. These results agree with results obtained in other studies on the antibacterial activity of the essential oil of *P. dioica* leaves (Vázquez-Cahuich *et al.,* 2013) and berries essential oil (Dussault *et al.,* 2014) were evaluated.

Minimum inhibitory concentration (MIC)

The microdilution in the plate is a reliable antimicrobial activity determination technique. Moreover, as in the disc diffusion technique results, the Gram-positive bacteria were more sensitive to the three *P. dioica* extracts evaluated, with MIC of 12.5 mg/mL, and had bactericidal activity. Meanwhile, Gram-negative bacteria had MIC of 25 mg/mL, and had bacteriostatic activity, except for AE which inhibited *E. coli* growth with MIC of 12.5 mg/mL. WE had a lower antioxidant activity than the EE and AE; however, WE had an antibacterial activity similar, and even higher against *E. coli*, which could be due to the presence of non-phenolic compounds with an antibacterial capacity to inhibit *E. coli* (Cowan, 1999). The different sensitivities of Gram-negative and positive bacteria to the *P. dioica* extracts were mostly attributed to the different cell wall structures of these two bacteria types. The cell wall of Gram-negative bacteria is made up of an external layer of lipopolysaccharides and an additional membrane, which can act as a protective barrier against hydrophobic compounds, including some phenolic and non-phenolic compounds contained in plant extracts.

HPLC analysis

Compounds found in the extracts

Using HPLC analysis, it was possible to

Table 4. Antibacterial activity of *P. dioica* leaves crude extracts.

Extract	Gram-positive				Gram-negative			
	***Bacillus cereus* (ATCC 11778)**		***Staphylococcus aureus* (ATCC 25923)**		***Salmonella Typhimurium* (ATCC 14028)**		***Escherichia coli* (ATCC 35150)**	
	DD	**MIC**	**DD**	**MIC**	**DD**	**MIC**	**DD**	**MIC**
Aqueous	13.5 ± 1.7^{a}	12.5	15.3 ± 2.4^{a}	12.5	8.8 ± 1.9^{a}	25	9.3 ± 2.6^{a}	12.5
Ethanolic	10.0 ± 0.8^{b}	12.5	12.0 ± 3.6^{ab}	12.5	8.0 ± 0.8^{a}	25	7.5 ± 0.6^{a}	25
Acetonic	12.0 ± 2.3^{a}	12.5	8.3 ± 0.5^{b}	12.5	7.8 ± 1.3^{a}	25	9.0 ± 1.2^{a}	25

Values are means ± standard deviations of triplicates (n = 3). Means in the same column followed by a different superscript lowercase letter are significantly different ($p < 0.05$). DD = disc diffusion, zone of inhibition reported in mm; and MIC = Minimum Inhibitory Concentration, expressed in mg/mL.

detect and confirm the presence of phenols, triterpenes, sapogenins, and polyalcohols in WE, EE, and AE crude extracts of *P. dioica* leaves. It is important to mention that some of the components found by HPLC could not be identified; however, the presence of phenolic compounds in the components identified was notable. Table 5 lists the components found with their respective formula, molecular weight (m/z), and retention time (t_R). In WE, seven phenolic compounds were identified: gallic acid, kaempferol, quercetin, catechin, caffeic acid, chlorogenic acid, and (-)-epicatechin. In EE, ten compounds were identified, of which seven were phenols or flavonoids (gallic acid, myricetin, peduncalagin, epicatechin-3-O-gallate, kaempferol, and quercetin), two are triterpenoids (brachycarpone and glaucalactone), and one was sapogenin (hecogenin acetate). In AE, a phenolic compound (catechin), a polyalcohol (pentaeythriol tetrapropionate), and a sapogenin (hecogenin acetate) were identified.

The studies carried out on *P. dioica* leaves are few, and generally carried out on the berries. Any works carried out with *P. dioica* leaves are often done on essential oils. One of the few papers that reported the chemical components of crude extracts of *P. dioica* was carried out by Onwasigwe *et al.* (2017). They prepared aqueous and methanol extracts and reported the presence of eugenol, quercetin gallic acid, and ericifolin. On the other hand, phenolic compounds found in the present work have a wide range of pharmacological activities including antioxidant, anti-inflammatory, antimicrobial, anticancer, cardioprotective, neuroprotective, antidiabetic, anti-osteoporotic, estrogenic/antiestrogenic, anxiolytic, analgesic, and anti-allergic activities (Li *et al.,* 2016; Naveed *et al.,* 2018; Imran *et al.,* 2019). In *P. dioica* berries, the presence of terpenoids has been reported during extraction with a solid-phase microextraction fibre system (Bajer *et al.,* 2016). The compound brachycarpone (trinortriterpenoid) was initially isolated from *Cleome branchycarpa*, a plant used for the treatment of scabies, rheumatism, and inflammation (Ahmad *et al.,* 1986). Glaucalactone (terpenoid) has antidiabetic effects (Mpetga *et al.,* 2014), and sapogenin (hecogenin acetate) has been used as an analgesic (Quintans *et al.,* 2017). There have been a

Table 5. Compounds found in the extracts (aqueous, ethanolic, and acetonic) of leaves of *P. dioica.*

Extract	Compound	Formula	[M-H]- (m/z)	t_R (min)
Aqueous	Catechin	$C_{15}H_{14}O_6$	289.841	1.192
	Caffeic acid	$C_9H_8O_4$	179.0235	1.92
	(-)-epicatechin	$C_{15}H_{14}O_6$	291.032	2.01
	Gallic acid	$C_7H_6O_5$	169.0137	3.018
	Chlorogenic acid	$C_{16}H_{18}O_9$	355.029	4.653
	Kaempferol	$C_{15}H_{10}O_6$	285.0399	11.567
	Quercetin	$C_{15}H_{10}O_7$	301.0348	13.488
Ethanolic	Gallic acid	$C_7H_6O_5$	169.0137	3.01
	Myricetin	$C_{15}H_{10}O_8$	317.0297	6.148
	Ellagic acid	$C_{14}H_6O_8$	300.9984	6.954
	Peduncalagin	$C_{34}H_{24}O_{22}$	783.0681	7.237
	Epicatechin-3-O-gallate	$C_{22}H_{18}O_{10}$	441.0822	9.569
	Kaempferol	$C_{15}H_{10}O_6$	285.0399	11.567
	Quercetin	$C_{15}H_{10}O_7$	301.0348	13.479
	Brachycarpone	$C_{29}H_{44}O_6$	487.3060	18.21
	Hecogenin acetate	$C_{29}H_{44}O_5$	471.3110	22.72
	Glaucalactone	$C_{29}H_{44}O_4$	455.3161	27.40
Acetonic	Catechin	$C_{15}H_{14}O_6$	289.841	1.192
	Pentaerythritol tetrapropionate	$C_{17}H_{28}O_8$	359.1706	14.19
	Hecogenin acetate	$C_{29}H_{44}O_5$	471.3110	22.47

few reports on pentaerythritol tetrapropionate polyalcohol, and it has been identified as an antioxidant (Machtin *et al.,* 1981).

Conclusions

Pimenta dioica leaves are an important source of phenolic compounds, many of which have important biological activities. The antioxidant property of the acetonic extract of *P. dioica* leaves was apparent. The three extracts (aqueous, ethanolic, and acetonic) of *P. dioica* leaves had a high total phenolic content, and showed important antioxidant activity. Therefore, *P. dioica* leaves, which are crop residues, have a potential as an antioxidant agent to be used in the food, pharmaceutical, and cosmetic industries. Extracts of *P. dioica* leaves had no antibacterial activity against Gram-negative bacteria (*S.* Typhimurium and *E. coli*), but showed bacteriostatic activity against Gram-positive bacteria (*S. aureus* and *B. cereus*). More research are required to evaluate the potential use of *P. dioica* leaf extracts as antimicrobial agents.

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