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TECHNICAL ARTICLE

Parastrephia quadrangularis: A Possible Alternative to Inhibit the Microbial Effect on the Generation of Acid Mine Drainage

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Abstract
The role of iron- and sulfur- oxidizing microorganisms in the generation of acid mine drainage (AMD) from sulfide ores and tailings is widely recognized. The inhibition of these microorganisms is a very important alternative to mitigate AMD formation and its effects on the environment and ecosystems. *Parastrephia quadrangularis* (commonly known as Tola–Tola) is a xerophytic plant that usually grows very close to or even on mineral processing tailings located in the Puna region of Argentina. We demonstrate that the aqueous extract of *P. quadrangularis* is capable of inhibiting the growth of *Acidithiobacillus ferrooxidans* in suspension. The presence of chlorogenic acid, quinic acid, butyl-1caffeoylquinic, ferulic acid, and euparin, which have already demonstrated antimicrobial action, was detected. The inhibition of *A. ferrooxidans* could be the basis of a possible alternative to inhibit microbial activity in tailings and thus prevent AMD.

Keywords *Acidithiobacillus ferrooxidans* · Native plants · Aqueous extract · Antimicrobial action · Tailings

Introduction

Acid mine drainage (AMD) is an acidic metal-rich water resulting from the exposure of sulfide minerals to air and water that can occur in operating and abandoned mines. Although the ecology of microbial communities in AMD is complex (Baker and Banfield 2003; Castro et al. 2019; Nyström et al. 2019), *Acidithiobacillus ferrooxidans* is probably the species more frequently found in such habitats (Cazón et al. 2013; Sajjad et al. 2018; Yang et al. 2014;). Since this mesophilic species is an iron- and sulfur-oxidizer, it can be seen as the representative microorganism in these communities.

Due to the role of *A. ferrooxidans* (and other acidophilic microorganisms), controlling and inhibiting its activity is an effective strategy to reduce AMD formation. Based on this concept, bactericides such as anionic surfactants, organic acids, and food preservatives have been used to inhibit the growth of these microorganisms. Acidophiles thrive under highly acidic conditions (< pH 3) but to survive, they require an intracellular pH between 6.5 and 7.0, which they achieve by restricting proton entry into the cell via the cytoplasmic membrane. However, anionic surfactants allow protons to penetrate freely into the bacteria's cell membranes, causing disruptions of enzymatic functions at low concentrations and even cell death at higher concentrations (Zhang and Wang 2017). In addition to anionic surfactants, different organic acids have been used (e.g. acetic acid, lactic acid) that are harmful to acidophiles because, under acidic conditions, they decouple the respiratory chain by penetrating the cell membrane of their protonated forms; these species deprotonate inside the cell, releasing protons that affect different cell functions and can cause cell death (Baker-Austin and Dopson 2007). Examples of the efficient use of these bactericides include the following: sodium lauryl sulfate (SLS) achieved a 60–95% decrease in acidity generation and a 90-95 % decrease in iron concentration in an acid drainage (Kleinmann and Erickson 1983); sodium dodecyl sulfate (SDS) inhibited iron oxidation in an *A. ferrooxidans* culture by almost 80% (Zhang and Wang 2017). Zhao et al. (2015) showed that the addition of furanone in very low concentrations (0.1–0.5 μM) inhibited the formation of biofilms in *A. ferrooxidans* cultures.

Similarly, organic acids are harmful to acidophiles because they uncouple the respiratory chain of these microorganisms under acidic conditions via the penetration of their protonated forms through the cell membrane, which

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then deprotonates while inside the cells, releasing harmful hydrogen ion (Baker-Austin and Dopson 2007). Until now, most bactericides studied for the inhibition of oxidizing iron-sulfur microorganisms are of chemical origin.

High concentrations of chloride have also been used to achieve microbial inhibition. Most acidophilic microorganisms have a high sensitivity to this anion (Ballerstedt et al. 2017). Bomberg et al. (2018) used natural saline water from the Rudna Mine to inactivate oxidizing iron microorganisms remaining in the reactor after the ore leaching process ends, i.e. in the post-mine operation stage. However, not all acidophiles are inhibited by salinity and some can cope with the osmotic stress and resist moderate concentrations, i.e. *Acidimicrobium ferrooxidans* and *A. caldus* (Zammit et al. 2012), while other halotolerants, such as *Acidihalobacter prosperus*, tolerate chloride concentrations up to 35 g/L (Nicolle et al. 2009).

Although some of these bactericides have a significant effect at very low concentrations, their use in the field requires transporting them to usually remote places. Also, they are soluble in water and can be dispersed by rainwater, requiring periodic reapplications. For example, Parisi et al. (1994) reported the need to spray sodium dodecyl benzene sulfonate four times a year on mining wastes to limit acidity generation and reduce effluent Fe and Mn concentrations by 82 and 90%, respectively.

The use of ubiquitous natural substances for the same purpose could be enormously advantageous. In this context, the microbial inhibitory capacity of many bioactive compounds extracted from Puna plants have been reported. The main hypothesis of our studies is that some of these compounds could also be inhibitory to the growth of iron- and sulfur-oxidizing microorganisms.

Parastrephia sp. is the most important genus growing in Argentina's highlands, the landscape being characterized by these perennial plants (Novara 2003). One of the species within this genus, *P. quadrangularis* (commonly known as Tola–Tola), usually grows very close to or even on mineral processing tailings. Only alcoholic extracts of this plant have been characterized (Gajardo et al. 2016; Palavecino-Ruíz et al. 2016). This resinous plant has different medicinal and pharmacological applications (Ardiles et al. 2018; Benites et al. 2012; Echiburu-Chau 2017; Gajardo et al. 2016; Rojo et al. 2009; Torres-Carro et al. 2015, 2017) and is also used for cattle feeding. Like other species belonging to the same genus, *P. quadrangularis* extracts have some antimicrobial and antifungal activities (Céspedes et al. 2015; Di Ciaccio et al. 2018; Palavecino-Ruíz et al. 2016; Sayago et al. 2012; Zampini et al. 2009).

To our knowledge, there are no reports in the literature on the compounds that could be released by the biomass of these plants exposed to rainfall. There are also no reports on the potential application of *P. quadrangularis* to inhibit the microorganisms that play a relevant role in the generation of AMD and whose main exponent is *A. ferrooxidans*. That is why, with the objective of analyzing its possible field application to mitigate the generation of AMD, we investigated the possible inhibitory action of an aqueous extract from *P. quadrangularis* on *A. ferrooxidans* growth in suspension. In addition, we analyzed the aqueous extract in order to suggest structures for the active compounds.

Materials and Methods

Study Site

The Puna plateau region is located in northwest Argentina between 3700 and 4500 m above sea level (masl), highly exposed to ultraviolet radiation, and extremely arid with an annual precipitation of 350 mm. Rainfall is strongly seasonal, with about 70% of the year's total rainfall occurring during summer (December to March), followed by 8 months of dry weather. The annual average temperature is 3–13 °C with high daily thermal fluctuations.

Numerous active and abandoned mines are located in this region and AMD has been detected in the tailings dams of many of these mines (Kirschbaum et al. 2012; Cazón et al. 2013; Murray et al. 2014, 2019). Mina Concordia is located in the province of Salta, Los Andes department, 15 km from the town of San Antonio de los Cobres, at an average altitude of 4200 masl. Exploitation began in 1900 and in 1986, mining and production of Ag, Pb, and Zn definitively stopped. The workings consisted of seven levels of galleries. It has four tailings dams located in a staggered and successive manner downstream on the right bank of the Concordia stream. Currently, the tailings deposits cover a total area of 8,537 m^2, with a calculated volume of 17,074 m^3. AMD can be detected in the tailings with pH values between 3.25 and 3.52 and containing high concentrations of Zn, Pb, Fe, Cu, and As.

Plant Material

The sampling campaign was carried out in October 2019 in the area around the abandoned Concordia mine (24° 12×28″ S and 66° 24×18″ W), Department of Los Andes, in Salta Province, at an altitude of 4200 masl. An area of 25 m^2 was delimited close to each of the four tailing dams. The aerial part of all *P. quadrangularis* plants (7–10 specimens) present in these areas was collected.

Extraction Procedure

The compounds present in the aqueous extracts of that plant (uncharacterized until now) could be representative of those

released when its biomass is exposed to rainwater. This is the reason why water was selected as the solvent. The elevated temperature (100 °C) used during the extraction (higher than the biomasses could be exposed to in the field) was chosen only to increase the extraction kinetics.

Biomasses collected were mixed and powdered; the particle size fraction between 1.8 and 2.0 mm was selected. The material was washed with deionized water several times and dried in an oven at 60 °C for 48 h. The following procedure was performed in triplicate. Ten grams of the dried material was extracted with distilled water (100 mL) at 100 °C for 1 h. The extract was initially filtered through Whatman paper, then through 0.45 and 0.22 µm membranes, and finally it was concentrated in a rotary evaporator (TE211 Evaporado Rotativo Tecinal) at 45 °C for 1 h. The gum obtained was dissolved in 100 mL of water, reaching a concentration of 12.9 mg/mL and a pH of 4.3. This aqueous extract suspension (AES) was stored until use in caramel-colored flasks at 4 °C. To study the effect of temperature on the extraction, a procedure similar to the one described above was carried out at room temperature instead of at 100 °C.

High-Performance Liquid Chromatography Analyses

High-performance liquid chromatography (HPLC) with a photo diode array (PDA) detector was used to determine the composition of the aqueous extract suspension (AES), and the collected fractions were analyzed by high-performance liquid chromatography-quadrupole mass spectrometry (HPLC-MS) to suggest structures for the active compounds.

Chromatographic Separation

Twenty µL of *P. quadrangularis* extract were injected using a Waters 717 autosampler injector. An isocratic flow rate of 0.8 mL/min was employed using a Waters 1525 binary pump, through a thermostatic column at 60 °C and with a carrier at pH 2.5 adjusted with formic acid. The flow was monitored at 254 nm, and the spectra of the selected peaks were analyzed with a Waters 2996 PDA detector, from 190 to 400 nm. A Gilson FC203b fraction collector was placed at the exit of the detector so that all of the eluent was fractionated in tubes, collecting 800 µL of each of the six fractions identified at 254 nm. These different fractions were later analyzed by HPLC-MS. Empower Pro software was used to analyze the extract spectra with a UV-visible spectral library from a software database.

Chromatographic Identification

The different fractions obtained during chromatographic separation were diluted in methanol:water with 0.1% formic acid (50:50 v/v) liquid chromatography–mass spectrometry (LC–MS) quality, and analyzed immediately after their preparation. The following conditions were used in the mass spectrometer: capillary voltage, 2.3 kV (ESI); cone voltage, 30 V; offset voltage, 80 V; source temperature, 120 °C; solvent gas temperature, 300 °C; solvent gas flow rate, 600 L/h; cone gas flow rate, 10 L/h. The mass spectrometer was calibrated in the *m/z* range 50–1200 using a 0.5 mM sodium formate solution prepared at 90:10 (v/v) 2-propanol:water. Data were corrected during acquisition using a reference compound (LockSpray). Data processing was performed using MassLynx v4.1 software (Waters Corp.). The spectrum of the sample/solvent mixture was obtained in the *m/z* range 100–650. The characteristic signals of each sample were extended, and the possible formulas of elemental composition were calculated in silico (tolerance: 5 mDa, fit conf.% > 10%) considering the elements C, H, O due to similarity to other existing analyses in the literature. On the other hand, for those samples whose MS/MS spectra had signals with higher intensity, it was possible to reach the probable molecular formula $C_xH_yO_z$ in which x, y, and z subscripts indicate numerical proportion of C, H, and O atoms respectively. Collision energies of 10, 20, and 30 V were used for these determinations.

Microbial Assays

Minimal Inhibitory Concentration

A collection strain of *A. ferrooxidans* DSM11477 was inoculated in 250 mL flasks containing 100 mL of 9 K medium (pH = 1.8); cultures were incubated at 150 rpm and 30 °C. When the exponential growth phase was reached, the cultures were filtered to remove jarosite and other solid-phase ferric compounds; the cells were then harvested and suspended again in an iron-free medium (0 K). To determine the minimal inhibitory concentration (MIC), 10 mL of that suspension (containing $\approx 1 \times 10^6$ cells/mL) was used to inoculate 90 mL of 9 K culture medium; different volumes (from 0.025 to 2.0 mL) of the AES (12.9 mg/mL) were added to the cultures. Therefore, the final concentration of the dry extract tested in the culture medium was between 0.0032 mg/mL and 0.258 mg/mL. Biotic (culture without the addition of extract) and abiotic (non-inoculated bottle with maximum extract concentration) controls were also performed. Cultures and controls were prepared in triplicate and incubated at 150 rpm and 30 °C. The addition of the extract produced undetectable changes in the pH of cultures and controls. Samples were taken every 4 h for 48 h. The concentration of ferrous iron was measured by titration with a standard solution of potassium permanganate. Microbial growth was indicated by the oxidation of ferrous iron, and MIC was defined as the lowest concentration of dried extract without microbial growth for 41 h.

Time for Irreversible Inhibition

To determine whether the inhibition observed in the previous experiments was reversible, *A. ferrooxidans* cultures were prepared in a similar way as described above and with the addition of the extract to reach the MIC (0.129 mg/mL). The flasks were incubated at 150 rpm and 30 °C. Samples were collected periodically to determine the pH, and the ferrous iron concentration was measured by titration with a standard solution of potassium permanganate. At different times, samples of the cultures were extracted and filtered through sterile 0.22 µm cellulose acetate membranes to retain the cells. The cell-laden membranes were then placed in flasks containing fresh 9 K culture medium without further inoculation and without a new addition of the extract. A biotic control was performed in parallel using cells not pretreated with the extract. Cultures and controls were followed through periodic determination of pH and ferrous iron concentration. The incubation time, in the presence of the extract at MIC, required to make the microbial inhibition irreversible was denominated TII (time for irreversible inhibition). TII was defined as the minimum time to cause total inhibition of microbial growth in the absence of the extract, for a minimum of 30 days.

Results and Discussion

Procedure to Obtain the Dry Extract of *Parastrephia quadrangularis*

Using the extraction protocol described above, 0.129 g of dry extract was obtained for each g of dry biomass treated. Extractions of bioactive compounds using water for this same plant were reported by Rojo et al. (2009), while others used other solvents (dichloromethane, ethyl acetate, *n*-hexane, methanol, chloroform) (Ardiles et al. 2018; Benites et al. 2012; Cifuentes et al. 2019; D´Almeida et al. 2012; Di Ciaccio et al. 2018; Gajardo et al. 2016; Torres-Carro et al. 2015, 2017; Palavecino-Ruíz et al. 2016) and methodologies (maceration, maceration-extraction using Soxhlet, extraction using Soxhlet). Higher yields (0.152, 0.5, 0.884 g of dry extract per g of dry biomass) but also lower ones (0.072 g of dry extract per g of dry biomass) were obtained (Ardiles et al. 2018; Benites et al. 2012; Gajardo et al. 2016; Torres-Carro et al. 2017). Water was used as the solvent in our extraction procedure so that we could evaluate the components that could be leached naturally (enabling its use in the field) rather than relying on organic solvents or more efficient extraction methodologies, which would not be economically feasible for field application.

Separation and Identification of the Aqueous Extract Components

Chromatographic separation of six different fractions was obtained. The retention times (t_r) for each of the collected fractions were 12.3, 19.2, 27.7, 36.9, 47.4, and 70.3 min (Fig. 1B), and the peaks in UV spectra for each collected fraction were 255.3, 321.9, 276.5, 308.7, 277.8, and 320.7 nm, respectively (Fig. 1A). The UV spectra of the fractions obtained were compared with the UV spectra database.

Each fraction was later analyzed using the Waters HPLC MS-MS system. In the case of fractions 1, 3, and 6, consistent structures were inferred from the molecular peak and the fragmentations. On the other hand, for fractions 2, 4, and 5 no conclusive structure could be established.

In the case of fraction 1 (t_r: 12.3 min), the structure obtained was that corresponding to euparin; in addition to the molecular ion ($[M-H]^-$: 217.0166), the spectrum presents the fragmentations (*m/z*: 160.089, *m/z*: 205.0709, and *m/z*: 137.0238) that justify the presence of euparin ($C_{13}H_{12}O_3$), which is a natural benzofuran (see Fig. 2). Natural benzofurans are heterocyclic compounds frequently synthesized by species of the family Asteraceae, and *Parastrephia* sp. belongs to this family (Visintini-Jaime et al. 2013). Euparin has also been found in other shrubs such as *Eupatorium buniifolium* (Visintini-Jaime et al. 2013). On the other hand, Benites et al. (2012) isolated and identified two other benzofurans (tremetone 1 and methoxytremetone 6) from *P. quadrangularis* but did not report the presence of euparin.

In fraction 3 (t_r: 27.7 min), the molecular ion corresponds to the compound butyl-1-caffeoylquinic ($C_{20}H_{26}O_9$) (Fig. 3A), while the fragments detected in HPLC-MS analysis were chlorogenic acid ($C_{16}H_{17}O_9$; *m/z*: 353.085) (Fig. 3B) and quinic acid ($C_7H_{12}O_6$; *m/z*: 191.945) (Fig. 3C). The compounds represented by those fragments have been identified in extracts from other plants, even in some species of the same genus as *P. quadrangularis*. For example, Abu-Reidah et al. (2013) found chlorogenic acid and quinic acid in the extract of *Cynara scolymus* L.; also, Echiburu-Chau et al. (2017) identified chlorogenic acid and quinic acid among 41 bioactive compounds found in the ethanolic extract of *P. lucida*.

In fraction 6 (t_r: 70.334 min), a molecular ion $[M-H]^-$ of 613.542 was determined with a fragmentation of ferulic acid ($C_{10}H_{10}O_4$; *m/z*: 193.05) (Fig. 4). Ferulic acid has been identified among 36 compounds in the ethanolic extract of *P. quadrangularis* (Cifuentes et al., 2019).

The chromatograms for the extracts obtained using water at room temperature are included in Supplementary Fig S-1; the peaks were similar to those obtained at higher temperature, although with much lower intensity. These results indicate that even at normal temperatures, the same compounds

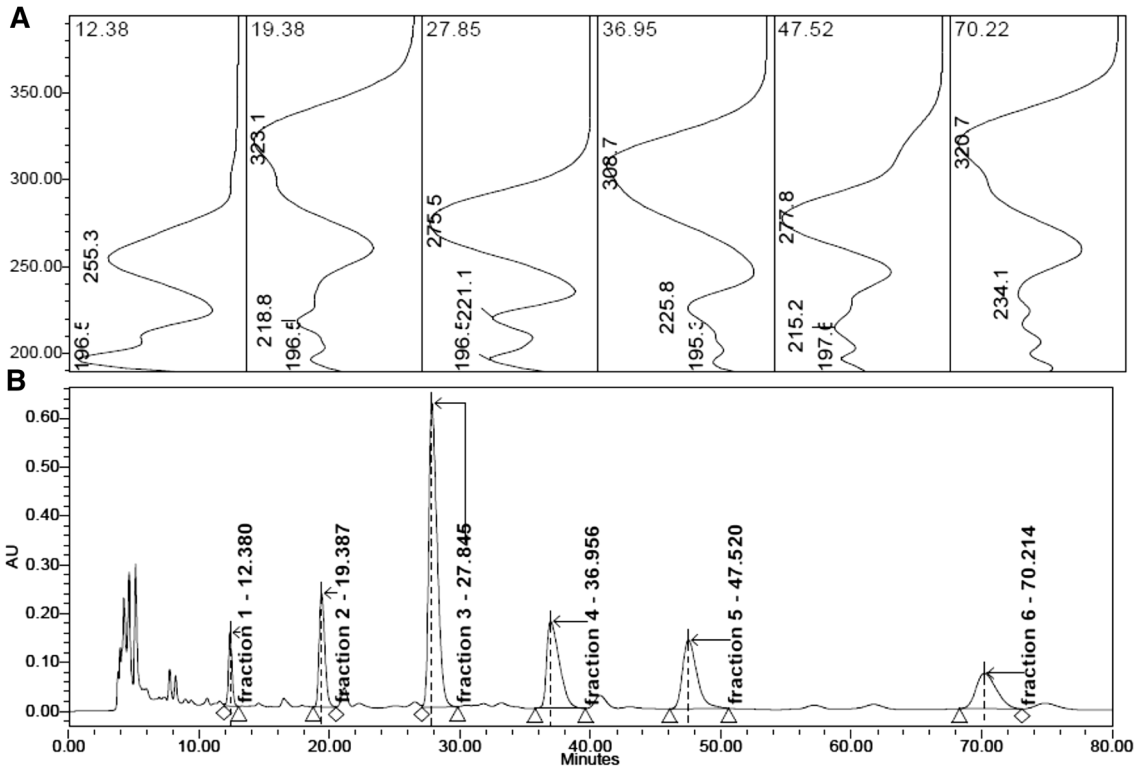

Fig. 1 HPLC-PAD chromatograms: **A** UV at 254 nm; **B** total ion current

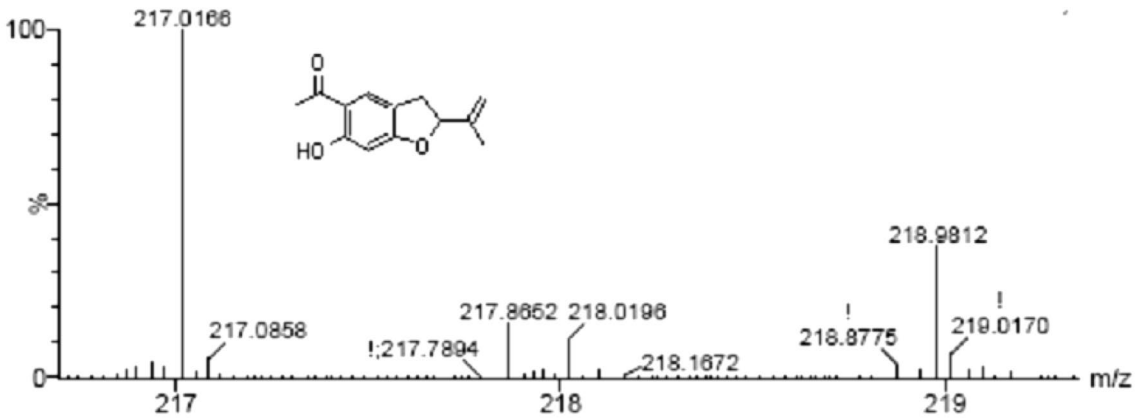

Fig. 2 HPLC-MS analysis and euparin structure

can be released by the extraction of *P. quadrangularis* biomass, although at a lower rate.

Microbial Assays

The addition of AES negatively affected the *A. ferrooxidans* cultures, reducing the rate of ferrous iron oxidation, which is a measure of microbial growth for this iron-oxidizing bacterium. Adding the extract did not modify the pH of the cultures and controls and the evolution of the controls with and without the addition of the extract was similar, so the partial or total inhibition was not due to changes in the pH.

Figure 5 shows the percentage of residual ferrous iron after the 41 h that the control without AES demanded to oxidize 9 g/L of ferrous iron that was originally present in the medium used (9 K). The MIC determined was 0.129 mg AES per mL of culture; that concentration was sufficient to cause inactivation of *A. ferrooxidans* growth after 41 h of incubation. Cultures with at least 0.129 mg of AES per mL showed no microbial growth or ferrous iron oxidation up to

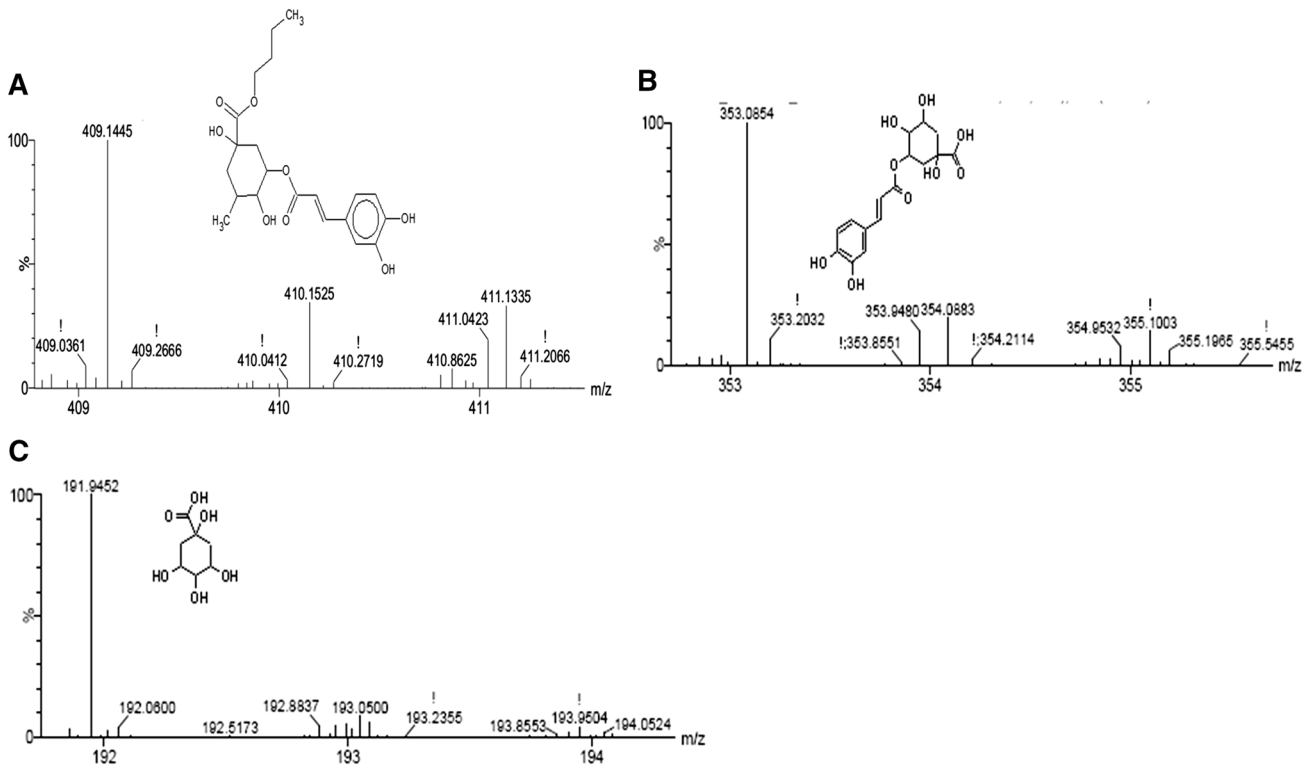

Fig. 3 HPLC-MS analysis. **A** Butyl-1caffeoylquinic structure; **B** chlorogenic acid structure; **C** quinic acid structure

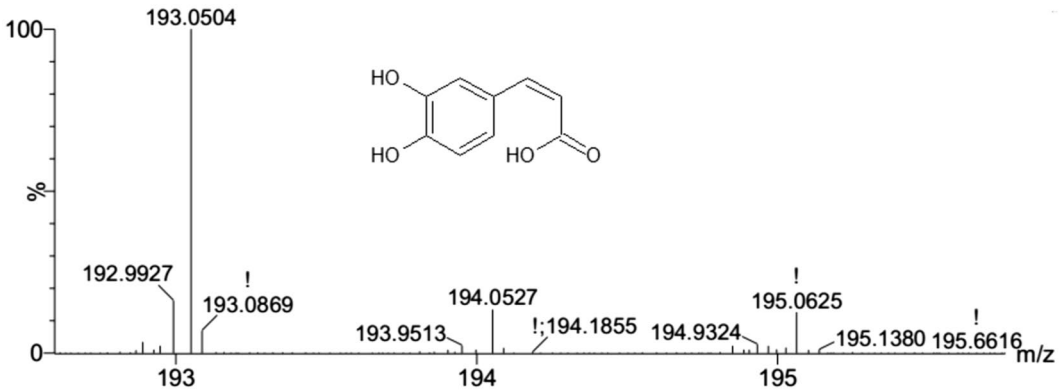

Fig. 4 HPLC-MS analysis and ferulic acid structure

100 h, similar to what was observed in the abiotic controls. No measurements were made after 100 h because incipient ferrous iron oxidation was observed in the abiotic controls after that time.

Time for irreversible inhibition (TII) was determined in other series of experiments where cells that were in a culture with the MIC for different lengths of time were then inoculated in fresh medium without AES. Cells that were in a culture with the MIC for only 30 min were able to oxidize all the ferrous iron at a similar rate to the biotic controls. On the other hand, cells that were in contact with the MIC at least for 15 days were not able to oxidize ferrous iron when inoculated in fresh medium and without AES in at least 30 days of incubation (not controlled afterwards). The obtained TII means that if *A. ferrooxidans* cells are in contact 15 days or more with AES, they will probably be inhibited for a long time even if they are no longer in contact with AES. This result could be important for possible use in the field where the extract could be washed out by rainfall. A previous and not so prolonged contact with the extract will maintain the

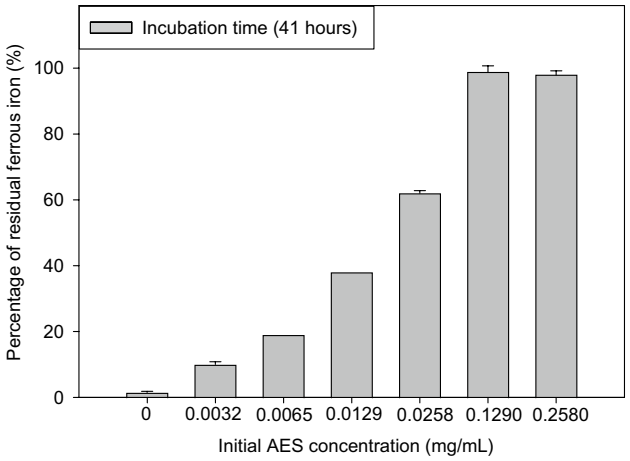

Fig. 5 Percentage of residual ferrous iron in *A. ferrooxidans* cultures with different AES (aqueous extract suspension of *P. quadrangularis* biomass) addition (x-axis represents the concentration of AES present in each culture). Bars and error bars correspond to the mean and average deviation obtained for three replicates

inhibition and consequently significantly reduce the risk of AMD generation without requiring the continuous addition of AES.

Although there are no previous reports on the use of *P. quadrangularis* extract to inhibit *A. ferrooxidans* growth, bioactive compounds of some species of the genus *Parastrephia* have been used in different applications within the area of medicine and agronomy (Ardiles et al. 2018; Cifuentes et al. 2019; Echiburu-Chau et al. 2017; Gajardo et al. 2016; Rodrigo et al. 2010; Rojo et al. 2009; Sayago et al. 2012; Torres-Carro et al. 2015, 2017; Zampini et al. 2009). For example, D'Almeida et al. (2012) showed that the extract of *P. lucida* (Meyen) has anti-inflammatory and antiseptic effects and that it is used in traditional medicine in northwestern Argentina. Specifically, the aqueous, methanolic, and dichloromethane extracts showed inhibitory effects on multi-resistant clinical isolates and also on fungi isolated from clinical samples. The reported MIC values ranged from 0.1 to 0.4 mg/mL for both bacteria and fungi. In a later work D'Almeida et al. (2013) investigated the effect of *P. lucida* extract on the enzymes involved in the biosynthesis of pro-inflammatory mediators of arachidonic acid. They found that inhibition was higher with the organic extracts (methanol and dichloromethane) than with the aqueous extract. Palavecino-Ruíz et al. (2016) investigated the ethanolic extract of *P. quadrangularis* as a fungicide on *Penicillium digitatum* and *Geotrichum citri-aurantii*, which affect lemon fruits after harvest. The MIC was 0.15 mg/mL while the minimum fungicide concentration was 0.350 mg/mL, sufficient for irreversible inhibition after 72 h (or 0.700 mg/mL for a time of 12 h). Di Ciaccio et al. (2018) studied the fungicidal action of *P. quadrangularis* extract on *Fusarium verticillioides*, which attacks corncobs in northern Argentina. Different MICs (0.118 and 0.250 mg/mL) were determined according to the origin of the plant from which the extract was obtained; this difference was attributed to the physiological stage of the plant at the time of collection and to the characteristics of the sampling site.

Other Puna plants have also shown antimicrobial activity. For example, Zampini et al. (2009) studied the antimicrobial activity of several species of plants from the Argentinian Puna on sensitive and multi-resistant Gram(+) and Gram(−) bacteria. Extracts of *Chuquiraga atacamensis* and plants of species of the genus *Parastrephia* were more effective, while those of *Fabiana species*, *Frankenia triandra*, *Chiliotrichiopsiskeidelii*, and *Tetraglochin cristatum* were the least effective. In general, aqueous extracts were less active than the ethanolic extracts. The MIC values were usually greater than those needed in this work to inhibit the growth of *A. ferrooxidans*.

Parastrephia quadrangularis is a native species in the Puna area where many abandoned and active mines are located, so it is readily available for possible use as an inhibitor of bioleaching microorganisms to stop the generation of AMD. In this work, we demonstrated that components in an aqueous extract of that plant have an inhibitory effect on planktonic cells of *A. ferrooxidans*. However, as previously stated, microbial communities in tailings are usually complex, including many species that are potentially AMD generators (Baker and Banfield 2003; Castro et al. 2019; Nyström et al. 2019); the action of AES will be effective in preventing AMD generation only if it is able to inhibit not only *A. ferrooxidans* but also the other leaching microorganisms, and not only in pure cultures but also in complex communities (Bernardelli et al. 2021; Cazón et al. 2021). Moreover, these microbial communities exist within biofilms in tailings and the action of biocides can be different on suspended or attached microorganisms (Zhao et al. 2015). Because of this, future work on the effect on *A. ferrooxidans* biofilms as well as other microbial species involved in AMD generation should be carried out.

Applicability to the Field Situation

The results presented in this study suggest that the use of *P. quadrangularis* biomasses could constitute a possible way to mitigate AMD generation. Since the La Puna geographic region has abundant rainfall in the summer season, compounds with inhibitory action could be released directly in the field. Thus, the placement of the biomass of that plant on tailing dams and other strategic places (where the presence of *A. ferrooxidans* and other bioleaching microorganisms has been detected) would allow the progressive extraction of their inhibitory components by the rainwater. This would partially or totally inhibit their bioleaching action and,

consequently, the generation of AMD. The results shown here are sufficiently novel (there is no precedent of similar studies for this or other plants) and of great accessibility (the plant is abundantly available in same area as many mining liabilities), so it is important to carry out new studies to analyze their eventual transfer to the field.

For the real applicability, it is necessary consider the field situation of many tailings dams. The main difficulty of a bactericide (either natural or of chemical origin) is ensuring that it contacts the microorganisms and can thus inhibit their activity. In the case of many tailings dam, there is a hard layer that hinders infiltration towards the interior; however, this layer usually cracks in drier seasons allowing water to flow towards the interior of the dam during rain events. In this way, the bactericide (the aqueous extract of *P. quadrangularis* in our case) could access internal areas of the tailings dam where AMD could be generated since they are precisely those that are exposed to water and oxygen that infiltrated. On the other hand, extracts can indeed be washed away during rain events, so it would be important that extraction of the inhibitory compounds be slow and continuous, which means that it is essential to retain the biomass on the dams (a simple and cheap device for this purpose is currently being developed). In addition, if the concentration of the inhibitory compounds exceeds MIC and the contact with the microorganisms is relatively prolonged, our results suggest that the inhibition will be maintained even if the extract is removed.

To summarize, our strategy would be to take advantage of the fact that *P. quarangularis* plants grow very close to mine tailings and even on tailings. Only the aerial part of the plant would be collected, allowing the plant to continue growing; the collected biomasses would have to be placed in ways that allow contact with rainwater to dissolve the active compounds without having them be washed away.

Conclusions

This work demonstrated that the aqueous extract of *P. quadrangularis* can inhibit the growth of *A. ferrooxidans* in suspension. Although it could not be determined whether this inhibition was due to the extract as a whole or only to some of its components, the presence of chlorogenic acid, quinic acid, butyl-1caffeoylquinic, ferulic acid, and euparin, which have already demonstrated antimicrobial action, was detected. These results suggest that *P. quadrangularis* biomass could be used to inhibit the growth of *A. ferrooxidans* at tailings dams and thus avoid AMD generation. Future work is needed to determine the efficiency of this inhibition on other microbial species and on the natural biofilms of these microorganisms, and to test this potential prevention and remediation method directly in the field.

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