**SUPPLEMENTARY MATERIAL**

**Green ultrasound-assisted extraction of antioxidant substances from the lichen *Hypotrachyna cirrhata*. Ethyl lactate, a better extracting agent than methanol toxic organic solvent?**

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**Abstract**

For the first time, we report a green extraction of lichen substances assisted by high power ultrasounds from *Hypotrachyna cirrhata* using ethyl lactate. This sustainable alternative was comparable, both in isolation and detection of lichen substances, to methanol. In the metabolomic analysis, a total of 77 lichen substances were detected comprising depsides, depsidones, dibenzofurans, organic acids, and lipids. Although the UHPLC/ESI/MS profiles were similar, the antioxidant activity was higher for the ethyl lactate extract. Ethyl lactate can replace toxic organic solvents, such as methanol, in order to provide more sustainable green chemistry methods.

**Keywords**: alternative solvents; antioxidants; green chemistry; Hypotrachyna; LC/MS; lichens; Ultrasound

**Experimental**

***Chemicals***

UHPLC-MS solvents were purchased from Merck (Santiago, Chile). Ultrapure water was obtained from a Millipore water purification system (Milli-Q Merck Millipore, Chile). Ethyl lactate (CAS 687-47-8; bp: 154° C) and methanol were purchased from Sigma-Aldrich (Santiago, Chile). MeOH was used as control (conventional technique), while ethyl lactate was employed as green solvent for the extraction of lichen substances (non-conventional technique).

***Lichen material***

*Hypotrachyna cirrhata* was collected at the river Huaura, Churin, Lima, Perú in October 2018 at 2258 m.a.s.l. The samples were identified by the lichenologist Daniel Ramos. The voucher herbarium specimens were kept at the Natural History Museum of the Universidad Nacional Mayor de San Marcos under reference number USM 294529.

***Maceration extraction***

The powdered sample of *H. cirrhata* (50 g) was placed in 300 mL of methanol at room temperature, and extracted for 48 h (3 times in the dark). After centrifugation (9000 g, 30 min), the supernatant was concentrated in vacuum to yield 2.3 g of a dark gummy extract (4.6%). For metabolomics studies were used 1.0 g of sample by triplicate under the same conditions mentioned (47.6±2.5 mg).

*Isolation*

This methanolic extract (2.3 g) was submitted to flash chromatography on silica gel (63-200 µm, 100 g, column length 25 cm, i.d. 10 cm) and eluted with n-hexane/EtOAc mixtures (2.0 L each) of increasing polarity (8:2, 6:4, 3:7, 0:1; v/v) to give four fractions.

Fraction 1 (0.7 g, n-hexane/EtOAc 8:2) was chromatographed on a SiO2 column (30 g) and eluted with n-hexane-EtOAc (1:0, 9:1, 7:3, 1:1 v/v) afforded 140 subfractions (20 mL each). These subfractions were combined based upon TLC monitoring obtaining three main fractions (1A -1C). Repeated CC (silica gel 63-200 µm, 30 g) on fraction 1A (0.2 g) eluted with n-hexane-EtOAc mixtures (0% - 10% EtOAc) led to the isolation of compounds **1** (protolichesterinic acid, 25 mg). CC on fraction 1B (0.3 g) eluted with n-hexane-EtOAc mixtures (0%-25% EtOAc) obtained compounds **1** (10 mg) and compound **2** (ergosterol peroxide, 5 mg). Fraction 1C (0.2 g) was submitted to Sephadex LH-20 (column length 40 cm, i.d. 6.5 cm, MeOH) and then to SiO2 CC to afford lipids according to 1H-NMR.

Fraction 2 (1.0 g, n-hexane/EtOAc 6:4) was subjected on Sephadex LH-20 using MeOH as mobile phase allowing the separation of fatty acids and chlorophylls affording two fraction (2A-2B). Fraction 2A (0.2 g) after repeated CC on silicagel using n-hexane-EtOAc mixtures (9:1, 8:2, 7:3, 6:4 and 1:1 v/v) gave compound **2** (30 mg). Further CC on Fraction 2B (0.1 g) was not observed the presence of secondary metabolites according to 1H-NMR

Fraction 3 (0.3 g, n-hexane/EtOAc 3:7) was passed on Sephadex LH-20 using MeOH as mobile phase and then was chromatographed on 20 g silica gel with n-hexane/EtOAc mixtures (0 up to 100%) affording compound **3** (2,4-dihydroxy-3-hydroxymethyl-6-methylbenzaldehyde; 5 mg).

Finally, Fraction 4 (0.3 g, n-hexane/EtOAc 0:1) was submitted to CC using Sephadex LH-20 (MeOH) allowing the separation of fats (discarded) again.

***Ultrasound-assisted extraction***

The powdered sample of *H. cirrhata* (50 g) was placed in 300 mL of ethyl lactate in the dark. The ultrasound-assisted extraction of the samples was performed in an ELMA ultrasonic bath (ELMA, GmbH, Germany) at 600 W and 35 kHz frequency for 30 min (The extracts do not increase in weight after this potency). After extraction, the supernatant was centrifuged for 30 min at 9000 g, and then, filtered and concentrated in vacuum below 50 °C to yield 3.2 g of a dark gummy extract (6.4%). For metabolomics studies were used 1.0 g of sample by triplicate under the same conditions mentioned (67±2 mg).

*Isolation*

The ethyl lactate extract (3.2 g) was submitted to flash chromatography on silica gel (63-200 µm, 130 g, column length 25 cm, i.d. 10 cm) and eluted with n-heptane/EtOAc mixtures (2.0 L each) of increasing polarity (8:2, 6:4, 3:7, 0:1; v/v) to give four fractions.

Fraction 1 (1.0 g, n-heptane/EtOAc 8:2) was chromatographed on a SiO2 column (40 g) and eluted with n-heptane-EtOAc (9:1, 7:3, 1:1 v/v) afforded 130 subfractions (20 mL each). These subfractions were combined based upon TLC monitoring obtaining three main fractions (1A -1C). Repeated CC (silica gel 63-200 µm, 30 g) on fraction 1A (0.3 g) eluted with n-heptane-EtOAc mixtures (0% - 10% EtOAc) led to the isolation of compounds **1** (protolichesterinic acid, 35 mg). Fraction 1B (0.4 g) eluted with n-heptane-EtOAc mixtures (0%-35% EtOAc) yielded compounds **1** (5 mg) and compound **2** (ergosterol peroxide, 15 mg). Fraction 1C (0.2 g) was submitted to 1H-NMR to afford lipids.

Fraction 2 (1.2 g, n-heptane/EtOAc 6:4) was subjected on CC on SiO2 affording two fraction (2A-2B). Fraction 2A (0.4 g) after CC on silicagel using n-heptane-EtOAc mixtures (9:1, 8:2, 7:3, 6:4 and 1:1 v/v) gave compound **2** (23 mg). Further CC on Fraction 2B (0.8 g) was not observed the presence of any secondary metabolites according to 1H-NMR.

Fraction 3 (0.8 g, n-heptane/EtOAc 3:7) was chromatographed on 30 g silica gel with n-heptane/EtOAc mixtures (0 up to 100%) affording compound **3** (2,4-dihydroxy-3-hydroxymethyl-6-methylbenzaldehyde; 25 mg).

Fraction 4 (0.2 g, n-heptane/EtOAc 0:1) showed the presence of fats according to 1H-NMR.

***UHPLC-PDA-MS Instrument***

The Thermo Scientific Dionex Ultimate 3000 UHPLC system hyphenated with a Thermo Q exactive focus machine used was already reported. For the analysis, 2 mg of each extract (all samples combined) were first dissolved in 2 mL of ethanol, then filtered (PTFE filter) and finally 10 µL were injected in the instrument, with all specifications set as previously reported (Calla-Quispe et al 2020).

***Extracts for UHPLC/DAD/ESI/MS/MS studies***

The dried lichen was ground by a hammer mill (Kinematica AG), filtered using a 0.2 mesh sieve, and then stored at -20°C. The extraction by maceration with methanol was carried out by adding 10 mL of solvent to 200 ± 0.5 mg lichen powder. Ultrasound-assisted extraction (UAE) was done under the same above-mentioned condition, using an US machine (ELMA, Germany) with the operating conditions being controlled (Frequency: 35 kHz; Power= 600 W, Time= 30 min and mode = sweep). Samples for each extract were carried out with 3 replications. The UHPLC/ESI/MS/MS analysis was done in negative mode for the optimal ionization of depsides and depsidones in this mode (Calla-Quispe et al 2020). Samples for metabolomics analysis were combined obtaining an extract by technique.

***LC parameters and MS parameters***

Liquid chromatography was performed using an UHPLC C18 column (Accucore, 150 mm ×4.6 mm ID, 2.5 µm, Thermo Fisher Scientific, Bremen, Germany) operated at 25 °C. The detection wavelengths were 254, 280, 330 and 354 nm, and PDA was recorded from 200 to 800 nm for peak characterization. Mobile phases were 1% formic aqueous solution (A) and 1 % formic acid in acetonitrile (B). The gradient program (time (min), % B) was: (0.00, 12); (5.00, 12); (10.00, 20); (15.00, 40); (20.00, 40); (25.00, 70); (35.00, 12) and 15 minutes for column equilibration before each injection. The flow rate was 1.00 mL min−1, and the injection volume was 10 µL. The standards, and the extracts dissolved in ethanol, were kept at 10 °C during storage in the autosampler. The HESI II and Orbitrap spectrometer parameters were optimized as previously reported (Calla-Quispe et al 2020).

***Compounds 1-3 isolated from Hypotrachyna extracts***

 

Figure S1. Compounds **1**-**3** isolated from *Hypotrachyna extracts*.

**Table S1. Identiﬁcation of metabolites in Peruvian lichen *Hypotrachyna cirrhata* by UHPLC-ESI-Q-Orbitrap-MS/MS.**

The qualitative analysis by UHPLC/ESI/MS/MS was performed for the characterization of secondary metabolites in complex mixtures, based on their full MS and MS/MS fragmentation patters (Calla-Quispe et al 2020). In the case of the molecular family of depsidones, the solvent ethyl lactate extracted 19 compounds, while methanol extracted 18 compounds. Peak 61, with a parent ion at m/z 357.0616 was identified as hyposalazinic acid based on diagnostic daughter ions at m/z 313.0716 and 179.0344, was the only difference. In relation to the family of depsides, both solvents extracted 12 metabolites. With respect to the lipid family, ethyl lactate extracted 17 compounds, while methanol extracted 23 compounds. In this case, methanol showed more tendency to extract polar lipids by being is a more polar solvent (more dielectric constant) (Vovers 2017). The same trend was observed in the case of the molecular family of organic acids. No difference was observed in the case of the aromatics, polyols and dibenzofurans extraction. Another important difference was observed in the case of the unknown compounds. The green solvent ethyl lactate extracted 15 metabolites, while methanol solvent solubilized 9 compounds. Our results indicate that the green solvent was more efficient for the extraction of these unidentified compounds than MeOH was. In this case, ethyl lactate showed more tendency to extract, due to its lower polarity, than methanol (Pereira et al 2011). No match was found for the unknown compounds based upon LC/MS/MS data and literature search.

For the first time, we report the use of UHPLC/ESI/MS/MS for the identification of minor compounds. This evidence implies that H. cirrhata produces a vast number of compounds never reported before. This, could be due to the different ecosystems in which they grow. Further studies should be undertaken to disclose their varied chemistry in different places and times.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Peak** | **Tentative identification** | **[M-H]-** | **Retention time (min)** | **Theoretical mass (*m/z*)** | **Measured mass (*m/z*)** | **Accuracy (ppm)** | **MS ions (ppm)** | **Lichen** |
|  | **Depsidones** |  |  |  |  |  |  |  |
| 9 | Consalazinic acid derivative I | C18H15O11 | 13.75 | 407.0614 | 407.0623 | -2,2 | 363.0731; 377.0361; 149.0241 | 1; 2 |
| 10 | Consalazinic acid derivative II | C18H13O12 | 13.84 | 421.0407 | 421.0417 | -2,4 | 393.0311; 227.0198; 195.0297 | 1; 2 |
| 11 | Consalazinic acid derivative III | C17H11O11 | 13.92 | 391,0301 | 391,0311 | -2,6 | 347.0411; 137.0241; 121.0287; | 1; 2 |
| 12 | Consalazinic acid | C18H13O10 | 14.20 | 389,0509 | 389,0519 | -2,6 | 371.0411; 345.0619; 327.0513; 227.0351; 121.0288; 309.0403; 253.0508; 209.0609 | 1; 2 |
| 13 | Consalazinic acid derivative IV | C17H11O10 | 14.56 | 375,0362 | 375,0362 | 0,0 | 137,0237; 331.0464; 153.0190 | 1; 2 |
| 15 | Consalazinic acid derivative V | C18H11O11 | 14.96 | 403,0301 | 403,0311 | -2,5 | 387,0363; 329,0305; 149,0239; | 1; 2 |
| 18 | Consalazinic acid derivative VI  | C17H11O9 | 16.29 | 359.0403 | 359.0408 | -1,4 | 315.0507 | 1; 2 |
| 21 | Consalazinic acid derivative V isomer | C18H11O11 | 16.61 | 403.0301 | 403.0304 | -0,7 | 329.0305; | 1; 2 |
| 22 | Consalazinic acid derivative VIII isomer | C19H13O11 | 16.98 | 417.0458 | 417.0462 | -1,0 | 371.0406; 387.0356; 177.0189 | 1; 2 |
| 23 | Salazinic acid | C18H11O10 | 19.03 | 387,0352 | 387,0362 | -2,6 | 343.0461; 299.0565; 151.0396; 325.0357 | 1; 2 |
| 26 | Salazinic acid I | C18H11O10 | 20.90 | 387.0352 | 387.0367 | -3,9 | 343.0466; 151.0397; 123.0445; | 1; 2 |
| 30 | Menegazziaic acid | C18H13O9 | 21.77 | 373.0560 | 373.0572 | -3,2 | 267.0667; 311.0565; 355.0464; 255.0674; 343.0456; 329.0679 | 1; 2 |
| 33 | Consalazinic acid derivative XIII | C19H11O11 | 22.13 | 415.0301 | 415.0312 | -2,7 | 375.0725 | 1; 2 |
| 36 | Salazinic acid II | C18H11O10 | 22.34 | 387.0352 | 387.0359 | -1,8 | 343.0459; 209.0560; 151.0393; | 1; 2 |
| 38 | Galbinic acid isomer | C20H13O11 | 22.43 | 429.0458 | 429.0468 | -2,3 | 123.0445; 269.0459; 149.0241 | 1; 2 |
| 40 | Constictic acid isomer | C19H13O10 | 22.56 | 401.0509 | 401.0514 | -1,2 | 357.0613; 313.0718; 121.0285 |  1; 2 |
| 42 | Norstictic acid | C18H11O9 | 22.85 | 371.0403 | 371.0414 | -3,0 | 327.0517; 227.0350; 243.0300; 151.0395; 283.0614; 255.0667 | 1; 2 |
| 58 | Psoromic acid | C18H13O8 | 24.38 | 357.0610 | 357.0622 | -3,4 | 313.0723; 269.0821; 181.0505; 179.0348; 327.0517; 285.0774 | 1; 2 |
| 61 | Hyposalazinic acid | C18H13O8 | 24.71 | 357.0610 | 357.0616 | -1,7 | 313.0716; 179.0344 | 2 |
|  |  |  |  |  |  |  |  |  |
|  | **Depsides** |  |  |  |  |  |  |  |
| 16 | Thamnolic acid | C19H15O11 | 15.57 | 419.0614 | 419.0626 | -2,9 | 209.0452; 167.0347 | 1; 2 |
| 17 | Haemathamnolic acid | C19H15O10 | 15.61 | 403.0665 | 403.0669 | -1,0 | 359.0772; 209.0449 | 1; 2 |
| 19 | Consalazinic acid derivative IX | C19H13O12 | 16.31 | 433.0407 | 433.0414 | -1,6 | 419.0261; ; 403.0311; 417.0468 | 1; 2 |
| 20 | Thamnolic acid isomer | C19H15O11 | 16.42 | 419.0614 | 419.0617 | -0,7 | 209.0450; 167.0343 | 1; 2 |
| 24 | Consalazinic acid derivative XII | C19H11O12 | 20.40 | 431.0251 | 431.0260 | -2,1 | 387.0364 | 1; 2 |
| 37 | Lecanoric acid isomer | C16H13O7 | 22.36 | 317.0661 | 317.0670 | -2,8 | 167.0347; 123.0446; 149.0240 | 1; 2 |
| 49 | Hypotrachynin B | C20H17O10 | 23.79 | 417.0822 | 417.0836 | -3,4 | 373.0948; 151.0398; 373.0545 | 1; 2 |
| 62 | 4-0-Demethylbaeomycesic acid isomer | C18H15O8 | 25.32 | 359.0767 | 359.0778 | -3,1 | 181.0503; 163.0397; 137.0603 | 1; 2 |
| 64 | Squamatic acid | C19H17O9 | 25.57 | 389.0873 | 389.0886 | -3,3 | 343.0827; 163.0398; 193.0141; 149.0240; 121.0288 | 1; 2 |
| 69 | Subsphaeric acid | C21H23O7 | 27.44 | 387.1444 | 387.1455 | -2,8 | 223.0978; 181.050; 343.0827 | 1; 2 |
| 74 | Hypotrachynic acid | C18H15O7 | 29.58 | 343.0818 | 343.0827 | -2,6 | 329.0623; 299.0931 | 1; 2 |
| 77 | Atranorin | C19H17O8 | 30.33 | 373.0923 | 373.0932 | -2,4 | 119.0494; 163.0397; 177.0191 | 1; 2 |
|  | **Lipids/oxylipins** |  |  |  |  |  |  |  |
| 25 | Hexahydroxytetracosanoic acid | C24H47O8 | 20.71 | 463.3271 | 463.3282 | -2,4 | 449.3123 | 1; 2 |
| 29 | Pentahydroxytetracosanoic acid | C24H47O7 | 21.64 | 447.3322 | 447.3333 | -2,5 | 433.3186; 389.2908 | 1; 2 |
| 32 | Hexahydroxyoxohexacosanoic acid | C26H49O9 | 22.02 | 505.3377 | 505.3386 | -1,8 | 445.3173; 433.3174 | 1; 2 |
| 39 | Pentahydroxytetracosanoic acid I | C24H47O7 | 22.54 | 447.3322 | 447.3333 | -2,5 | 433.3172; 389.2912 | 1; 2 |
| 41 | Tetrahydroxydocosanoic acid | C22H43O6 | 22.66 | 403.3060 | 403.3071 | -2,7 | --- | 1; 2 |
| 43 | Pentahydroxyhexacosanoic acid | C26H51O7 | 23.08 | 475.3635 | 475.3644 | -1,9 | 429.3224; 447.3336 | 1; 2 |
| 44 | Tetrahydroxydocosanoic acid I | C22H43O6 | 23.14 | 403.3060 | 403.3063 | -0,7 | --- | 1; 2 |
| 45 | Tetrahidroxytricosanoic acid | C23H45O6 | 23.17 | 417.3216 | 417.3228 | -2,9 | 399.3124 | 1; 2 |
| 46 | Pentahydroxyicosatrienoic acid | C20H33O7 | 23.36 | 385.2226 | 385.2239 | -3,4 | 329.2329; 357.2289 | 1 |
| 50 | Pentahydroxyhexacosanoic acid I | C26H51O7 | 23.85 | 475.3635 | 475.3645 | -2,1 | 429.3227; 447.3330 | 1; 2 |
| 51 | Hexahydroxyheptacosenoic acid | C27H51O8 | 23.89 | 503.3584 | 503.3596 | -2,4 | 475.3646; 443.3381 | 1; 2 |
| 52 | Tetrahydroxynonadecatrienoic acid | C19H31O6 | 23.92 | 355.2121 | 355.2133 | -3,4 | 311.2231; 181.1231 | 1; 2 |
| 54 | Methyl pentahydroxyoxoheptacosanoate | C28H53O8 | 24.15 | 517.3740 | 517.3748 | -1,5 | 457.3535 | 1; 2 |
| 55 | Trihydroxynonadecadienoic acid | C19H33O5 | 24.26 | 341.2328 | 341.2339 | -3,2 | 323.2232; 171.1021 | 1 |
| 59 | Tetrahydroxypentacosanoic acid | C25H49O6 | 24.52 | 445.3529 | 445.3542 | -2,9 | 417.3232 | 1 |
| 60 | Trihydroxynonadecatrienoic acid | C19H31O5 | 24.65 | 339.2171 | 339.2182 | -3,2 | 295.2282; 311.2233 | 1 |
| 65 | Tetrahydroxyicosatrienoic acid | C20H33O6 | 26.65 | 369.2277 | 369.2288 | -3,0 | 325.2388 | 1 |
| 72 | Tetrahydroxyoctacosahexaenoic acid isomer | C28H43O6 | 28.70 | 475.3060 | 475.3064 | -0,8 | --- | 1 |
| 35 | Jackinic acid | C19H33O6 | 22.33 | 357.2277 | 357.2290 | -3,6 | 313.2392; 325.2027 | 1; 2 |
| 48 | 6-ethyl-6-n-pentylpentadecan-4,5,7,8,15-pentol-15-acetate | C24H47O6 | 23.65 | 431.3373 | 431.3383 | -2,3 | 417.3228 | 1; 2 |
| 67 | Norcaperatic acid | C20H35O7 | 26.81 | 387.2383 | 387.2393 | -2,6 | --- | 1; 2 |
| 73 | Norcaperatic acid derivative II | C20H33O7 | 29.29 | 385.2226 | 385.2237 | -2,9 | 341.2337; 295.2273 | 1; 2 |
| 75 | Norcaperatic acid derivative II isomer | C20H33O7 | 29.75 | 385.2226 | 385.2238 | -3,1 | 341.2336; 295.2280 | 1; 2 |
|  | **Organic acids** |  |  |  |  |  |  |  |
| 2 | Ascorbic acid | C6H7O6 | 2.90 | 175,0243 | 175,0247 | -2,3 | 133,0501; 145,0138 | 1; 2 |
| 3 | Ascorbic acid isomer | C6H7O6 | 3.12 | 175,0243 | 175,0247 | -2,3 | 133,0501; 145,0138 | 1; 2 |
| 4 | Citric acid | C6H7O7 | 3.49 | 191,0192 | 191,0196 | -2,1 | 111.0081 | 1 |
| 5 | Arabic acid | C5H9O6 | 3.50 | 165.0399 | 165.0397 | 1,2 | 147.0291; 113.0234; 121.0183 | 1; 2 |
|  | **Dibenzofurans** |  |  |  |  |  |  |  |
| 76 | Usnic acid | C18H15O7 | 30.02 | 343.0818 | 343.0826 | -2,3 | 231.0607; 328.0593 | 1; 2 |
|  | **Aromatics** |  |  |  |  |  |  |  |
| 71 | Ethyl-4-O-methylolivetolcarboxylate | C15H21O4 | 28.55 | 265.1440 | 265.1481 | -15,5 | --- | 1; 2 |
|  | **Polyols** |  |  |  |  |  |  |  |
| 1 | Mannitol | C6H13O6 | 2.71 | 181,0712 | 181,0716 | -2,2 | 151,0607 | 1; 2 |
|  | **Unknown compounds** |  |  |  |  |  |  |  |
| 6 | Unknow | C17H11O12 | 13.13 | 407,0251 | 407,0261 | -2,5 | --- | 1; 2 |
| 7 | Unknow | C23H25O15 | 13.25 | 541,1193 | 541,1201 | -1,5 | --- | 1; 2 |
| 8 | Unknow | C26H21O13N | 13.34 | 555,1013 | 555,0997 | 2,9 | --- | 1; 2 |
| 14 | Unknow | C26H19O13N | 14.90 | 553,0856 | 553,0836 | 3,6 | --- | 1 |
| 27 | Unknow | C14H13O7 | 20.96 | 293.0661 | 293.0672 | -3,8 | --- | 1; 2 |
| 28 | Unknow | C20H13O12 | 21.02 | 445.0407 | 445.0419 | -2,7 | --- | 1; 2 |
| 31 | Unknow | C23H21O12 | 21.79 | 489.1033 | 489.1036 | -0,6 | --- |  2 |
| 34 | Unknow | C23H19O14 | 22.19 | 519.0775 | 519.0776 | -0,2 | --- |  2 |
| 47 | Unknow | C28H23O15 | 23.38 | 599.1037 | 599.1042 | -0,8 | --- | 2 |
| 53 | Unknow | C23H19O12 | 23.92 | 487.0877 | 487.0879 | -0,4 | --- | 2 |
| 56 | Unknow | C30H25O14 | 24.29 | 609.1244 | 609.1260 | -2,6 | --- | 1; 2 |
| 57 | Unknow | C23H9O4 | 24.34 | 349.0501 | 349.0491 | 2,9 | --- | 1; 2  |
| 63 | Unknow | C23H21O11 | 24.92 | 473.1084 | 473.1086 | -0,4 | --- | 2 |
| 66 | Unknow | C24H11O6 | 25.73 | 395.0556 | 395.0536 | 5,1 | --- | 2 |
| 68 | Unknow | C19H9O5 | 26.93 | 329.0450 | 329.0432 | 5,5 | --- | 2 |
| 70 | Unknow | C25H11O7 | 27.84 | 423.0505 | 423.0489 | 3,8 | --- | 1; 2 |

**Lichen 1**: Methanol extract; **Lichen 2**: Ethyl lactate extract

**Table S2. Metabolites isolated from Hypotrachyna genus.**

|  |  |
| --- | --- |
| **Species (From)** | **Metabolite** |
| *H. ikomae* (Malaysia) | 1-6 (Din et al 2010) |
| *H.imbricatula* (Malaysia) | 3,6,7-10 (Din et al 2010) |
| *H. toiana* (Malaysia) | 3, 6, 8-13 (Din et al 2010) |
| *H. chicitae* (Peru) | 3, 14 (Mandujano et al 2013) |
| *H. leiophylla* (South Africa) | 3, 6, 15-16 (Elix et al 2000) |
| *H. quaesita* (Papua New Guinea) | 3, 6, 17-20 (Elix et al 1999) |
| *H. caraccensis* (Colombia) | 14, 21-23 (Leal et al 2018) |
| *H. revoluta* (Turkey) | 3, 23, 24-30 (Papadopoulou et al 2007) |
| *H. hypoalectorialica* (Brazil) | 14, 31-33 (Elix et al 1996) |
| *H. partita* (Costa Rica) | 3, 34-35 (Culberson et al 1977) |
| *H. immaculate* (Australia) | 3, 6, 12-13, 36-40 (Elix et al 2004) |
| *H. livida* | 38, 40-41 (Huneck and Yoshimura 1996) |

**Legend. 1**: Nephrosterinic acid. 2: Isonephrosterinic acid. 3: atranorin. 4: Protolichesterinic acid. 5: Lichesterinic acid. 6: Chloroatranorin. 7: Norobtusatic acid. 8: 4-O-demethylbarbatic acid. 9: Obtusatic acid. 10: Barbatic acid. 11: Vioxanthin. 12: Pigmentosin A. 13: Skyrin. 14: Usnic acid. 15: 3-chloro-4-O-demethylmicrophyllinic acid. 16: 4-O-demethylmicrophyllinic acid. 17: Fumarprotocetraric acid. 18: quaesitic acid. 19: Salazinic acid. 20: Protocetraric acid. 21: Hypotrachynin A. 22: Hypotrachynin B. 23: Methylstictic acid. 24: Hypotrachynic acid. 25: Deoxystictic. 26. Cryptostictinolide. 27: 8’-methylconstictic acid. 28: 8’-methylmenegazziaic acid. 29: Stictic acid. 30: 8’-Ethylstictic acid. 31: Alectorialic acid. 32: 5,7-dihydroxy-6-methylphthalide. 33: Hypoalectorialic acid. 34: Perlatolic acid. 35: Anziaic acid. 36: Colensoic acid. 37: Norcolensoic acid. 38: Methoxycolensoic acid. 39: Physodic acid. 40: Lividic acid. 41: Methyllividic acid. 42: Methylphysodic acid.

The chemistry of Hypotrachyna genus has reported the presence of lipids, depsides, depsidones, dibenzofurans, and naphthoquinones, based on the isolation of their metabolites using classic chromatographic processes and chemical methods. According to scientific literature, Hypotrachyna species from different places showed a production of various metabolites, with no quimiotaxonomic patterns being found. Secondary metabolites and their biosynthetic pathways are often specific and restricted to taxonomically related organisms. In general, most lichens of this genus seem to be producers of atranorin and chloroatranorin related compounds, while other reported compounds could be inducible metabolites given the dynamic environment. The varied chemistry from Hypotrachyna species has been demonstrated using classic methods. Culberson et al., 1977 reported the presence of thermally induced chemical artifacts from H. partita. Among them: anziol, olivetolcarboxylic acid, olivetol, 4-O-methylolivetolcarboxylic acid and O-methylolivetol from anziaic acid and perlatolic acid were detected. According to our studies, no chemical artifacts were detected or produced by using ultrasound-assisted extraction, as suggested by UHPLC/MS/MS.

**UHPLC-ESI-Q-Orbitrap-MS/MS Chromatogram of the extracts of *H. cirrhata*.**



**Figure S2. UHPLC-ESI-MS-MS Chromatogram of methanolic extract.**



**Figure S3. UHPLC-ESI-MS-MS Chromatogram of ethyl lactate extract.**

**Table S3. Scavenging of the 1,1-diphenyl-2-picrylhydrazyl Radical (DPPH), radical, ABTS as Trolox equivalent antioxidant capacity, (ABTS), Ferric Reducing Antioxidant Power (FRAP), and Total phenolic content (TPC) of *H. cirrhata* (n= 5).**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Samples (% w/w) | DPPHa | FRAPb | ABTSa | TPCc |
| EL (6.4) | 105.7 ± 0.01 | 196.4 ± 0.00 | 69.28 ± 0.00 | 153.6 ± 0.06 |
| MeOH (4.6) | 348.8 ± 0.02 | 30.18 ± 0.00 | 81.92 ± 0.00 | 75.94 ± 0.06 |
| GA | 1.12 ± 0.01 | 885.70 ± 0.03 | 8.73 ± 0.0 | - |
| Q | 7.36 ± 0.01 | 564.20 ± 0.07 | 15.32 ± 0.0 | - |

aDPPH and ABTS expressed as IC50 (µg/mL); bFRAP expressed as μmol Trolox/g dry weight; cTPC expressed as μmol GAE/g dry weight. Values in the same row marked with the same letter are not significantly different (at p < 0.05).

***Polyphenol contents***

The analyses of total phenolic compounds (TPC) was based on Guerrero-Castillo et al., 2019. To some 12 μL of extract to be measured, 168 μL of the 1% Folin-Ciocalteu reagent (Merck, Santiago) were added to well of a microplate reader. The mixture was allowed to react for 5 min, then 120 μL of 10% sodium carbonate was added. The mixture was incubated at room temperature for 30 min in darkness. Absorbance was then taken at 765 nm using an UV-Visible multiplate reader. The obtained absorbance values were replaced in the equation of the standard curve of gallic acid (μmol/L). The content regarding phenolic compounds was then expressed as gallic acid micromoles per gram of dry weight (μmol GAE/ g extract).

***Antioxidant assays***

 *DPPH cation radical discoloration test.*

The capturing capacity of the DPPH• radical was evaluated by the decolorization method (Guerrero-Castillo et al 2019). Briefly, 9 μL of extract, (2 mg/mL), plus 341 μL of methanol DPPH solution (400 μM) were adjusted with the solvent methanol to an absorbance of 1.10 ± 0.02 at 517 nm. The mixture was homogenized using a vortex, allowed to react in the dark at room temperature for 20 min, after which time absorbance was measured at 517 nm in a Synergy HTX monochromator (Biotek, USA). The percentage of discoloration of the DPPH moiety was obtained by measuring the change in absorbance at 517 nm. A curve was prepared with different dilutions of the extract, and the results were expressed as IC50 in microg per mL.

*Bleaching test with the cationic radical ABTS• +*

The capturing capacity of the ABTS•+ radical was evaluated by the decolorization method developed by Guerrero-Castillo et al., 2019. The radical ABTS•+ is generated chemically by the oxidation of ABTS with potassium persulfate after 16 h of incubation at room temperature in the dark. Briefly, 27 μL of the extract to be measured, (2 mg/mL), was added to 273 μL of the previously prepared ABTS•+ solution (previously adjusted with 80% methanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm), to the well of the microplate (Synergy HTX, Biotek USA) and subsequently allowed to react in darkness at room temperature for 6 min. The absorbance was then measured at 765 nm and the values obtained converted to % inhibition of the ABTS• + radical and a curve was prepared with different dilutions of the extract, and the results were expressed as IC50 in μg/mL.

*Ferric Reduction Ability-Antioxidant Power Test (FRAP)*

For the FRAP test, the methodology was performed with slight modifications for use in a microplate reader (Guerrero-Castillo et al., 2019). Briefly, to 10 μL of the dissolved extract (2 mg/mL), 290 μL of the FRAP solution was added and mixed in the well of the microplate, allowed to react in the dark at room temperature for 5 min. The absorbance measurement of the colored Fe-TPTZ complex was performed at 595 nm. Absorbance values were replaced in the Trolox standard curve equation (μmol/L). The results were determined as equivalents of Trolox (TE), in Trolox micromoles per gram of dry weight (μmol Trolox/g dry weight).

***Statistical Analysis***

 The statistical analysis was carried out using the originPro 9.1 software packages (Origin lab Corporation, Northampton, MA, USA). The determination was repeated at least three times for each sample solution. Analysis of variance was performed using ANOVA. Significant differences between means were determined by Dunnett comparison test (p values < 0.05 were regarded as significant).

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