*Research article*

# Effect of sonication and pressing on recovery of compounds with antioxidant activity from leaves and bark of *Byrsonima crassifolia*

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***Abstract.*** One of the traditional techniques to extract metabolites is maceration with solvents assisted by mechanical-grinding. Recently, ultrasound has been proposed as a factor to improve extraction. However, information about ultrasound times necessary is insufficient. Additionally, the pressing method helps the solids recuperation, abating loss of extract. This work established an efficient extraction methodology of metabolites with antioxidant activity, combining the ultrasound assisting solvent extraction and pressing (UPAE). Leaves and bark of B. crassifolia, dried by forced air, ground and sieved to form a flour, were used to make the UPAE, the results of the yields were compared with a conventional maceration (95% ethanol, 1: 4 p / v, control). The assisted ultrasound time were 5, 20, 40 and 50 min. The separation was carried out by pressing in a glass syringe. Antioxidant activity was determined qualitatively by fine chromatography plates and quantitatively by spectrophotometry DPPH antioxidant assay at 517 nm. The UPAE method increased the total solids extract, without affect the antioxidant activity of compounds in leaves as in bark, since 5 and 20 minutes, respectively. Without a specific identification, it was evidenced that *B. crassifolia* produces a great variety of antioxidant compounds in both leaves and the bark, that polar as apolar type, which remain unchanged independently of ultrasound assisted time. With this method is possibly make extractions with small amounts of powdered sample (4 g of flour), which would be advisable in studies when the biological material is limited.

**Keywords**. Ultrasound assisted extraction, antioxidant activity, *Byrsonima crassifolia*, pressing, maceration.

***Resumen***. Una de las técnicas tradicionales para extraer metabolitos es la maceración con solventes, ayudado de molienda mecánica. Recientemente, el ultrasonido se ha propuesto como un factor para mejorar la extracción. Sin embargo, la información sobre los tiempos de ultrasonido necesarios es insuficiente. Además, el prensado ayuda a la separación del bagazo y disminuye la pérdida de extracto.

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Se estableció una metodología de extracción de metabolitos antioxidantes, combinando la extracción con solvente asistida por ultrasonido y prensado (UPAE). Hojas y corteza de *B. crassifolia*, secadas mediante aire forzado, molidas y tamizadas hasta formar una harina, se usaron para realizar la UPAE, los resultados de los rendimientos se compararon con una maceración convencional (etanol al 95%, 1: 4 p/v, control). Los tiempos de ultrasonido fueron: 5, 20, 40 y 50 min. La separación por prensado se realizó con una jeringa de vidrio. La actividad antioxidante se determinó cualitativamente por TLC y cuantitativamente mediante ensayo espectrofotométrico de DPPH• a 517 nm. El método UPAE aumentó la cantidad de sólidos totales extraídos, sin afectar la actividad antioxidante, en hojas o corteza, desde los 5 y 20 minutos, respectivamente. Sin una identificación específica, se evidenció que *B. crassifolia* produce una gran variedad de compuestos antioxidantes, del tipo polar y apolar, los cuales permanecen inalterados independientemente del tiempo de sonicación. Con este método es posible hacer extracciones con pequeñas cantidades de muestra pulverizada (4 g de harina), lo cual sería aconsejable en estudios cuando el material biológico es limitado.

**Palabras clave.** Extracción asistida por ultrasonido, actividad antioxidante, *Byrsonima crassifolia*, prensado, maceración.

## INTRODUCTION

*Byrsonima crassifolia*, commonly known as nanche, is a perennial tree native to Central America, which is highly appreciated for its fruits.[1,2] de Souza *et al.,* (2012) report it as an important resource of antioxidant agents.[3]

Generally, plants are an important source of bioactive principles and drugs precursor that are extracted by solvents.[4] However, this method has several disadvantages as solvents and high energy spends, long extraction times and obtaining a variable yield at the end process, which depends on plants type.

The maceration of the different parts of the plant is a traditional technique, which is a solid-liquid extraction. This extraction type can be improved by decreasing the particle size by mechanical grinding. Recently, solid-liquid extraction has been assisted by sonication, which is fundamentally based on breaking the cell walls of plants with ultrasound, improving the penetration of the solvent and the mass transfer through the cell membrane.[5]

The extracted metabolites are filtered by gravity and/or vacuum, however, there is little information about the required sonication times to provide a benefit in yield. Finally, pressing helps to physical refining by applying a compression force on the filtered cake, abating loss of extracts in the solids recuperation. This work, established an efficient methodology to extract metabolites with antioxidant activity, which combines the benefits of extraction with solvents and ultrasonic and pressing assistance extraction (UPAE).

## MATERIALS AND METHODS

### Chemicals

Ethanol (GOLDEN BELL reactivos, México) was used for the extraction by maceration and UPAE. The liquid-liquid extraction was performed with methanol (GOLDEN BELL reactivos, México) and hexane (MEYER, México). Acetone was used as part of the mobile phase of Thin Layer Chromatography (TLC) (Silica gel 60 $F_{254}$, MERCKMILLIPORE). The 2,2-Diphenyl-1-picrylhydrazyl (DPPH),

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(±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (TROLOX) and TWEEN 80 were obtained from SIGMA-ALDRICH (MO, USA). The ascorbic acid was purchased from MEYER (México). All solvents used in this work were technical grade.

Biologicals material preparation

Leaves and bark from *B. crassifolia* were collected during august 2017, in Tuxtepec, Oaxaca. The collected material was dried by forced-air at 0.0103 $m^3/s$ for approximately 48 hours at 33±2 °C, crushed and grounded to reduce the particle size in a domestic blender (Black & Decker, México), finally it was sieved with an 8 pores/cm screen until to obtaining a powdered sample (flour). The flour was stored at room temperature for later use.

Maceration of leaf and bark

To comparing extraction yields between methods, we was used a maceration as control. This was carried out exposing the flour with ethanol for 24 h at room temperature, Flour of leave and bark was mixed with 95% ethanol to 1:4 w/v. It was filtered through fine-sized pore filter paper and extraction yield was determined.[6]

$$\text{yield (\%)} = \frac{\text{extract weight obtained}}{\text{flour initial weight}} \text{x } 100 \quad (1)$$

Ultrasound assisted extraction and pressing (UPAE)

Extraction process was carried out at room temperature, leaves or bark flour was mixed with ethanol to 1:4 w/v. They were sonicated at different times (5, 20, 40 and 50 min) with 50 watts in an ultrasonic bath (BAKU BK-3550 Ultrasonic Cleaner). The extraction product was recovered using a pressing process with a 50 mL glass syringe and a fine pore size filter. The bagasse was recovered and dried to constant weight to realize a mass balance (data not shown), finally it was eliminated, the extract was recovered and dried to constant weight.

The extract was concentrated through solvent evaporation using a simple distillation kit (GL207, GLASSCO, MÉXICO). The extracted solids yield was calculated using (1).

Liquid-liquid extraction and TLC separation

The ethanolic extract was dissolved in methanol (10 mg/mL) and it was mixed with hexane 1:1 v/v to carry out the liquid-liquid extraction. The compounds obtained were resolved in a thin layer chromatography (TLC), in a semi-hermetic environment using the acetone-methanol-ethanol-water system (1:2:1:2 v/v) as mobile phase, two known antioxidant compounds were used as controls: ascorbic acid and Trolox.

After running the plates, there were looked under UV light (365 nm), later there were sprayed with a DPPH solution and they were observed at 365 nm, again. Plates were photographed (12 megapixels, Dual Autofocus Pixel, f/1.7, LED flash) in each condition for digital analysis.

Analytical methods

Semiquantitative determination of antioxidant activity in thin layer chromatographic (TLC) plates

TLC plates were injected either, with a known concentration of leaf or bark extract obtained from assisted sonication at different times, or different concentrations of ascorbic acid (10, 5, 0.5, 0.05 y 0.005 mg/mL). Later, TLC plates were sprayed with a methanolic DPPH solution (0.15% w/v), and then were photographed to digital analysis.[7]

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Spectrophotometric determination of DPPH antioxidant assay

Antioxidant activity was measured according to Sharma and Bath (2009).[8] 200 µL of stock solution (10 mg/mL) of each extract were taken, which were mixed with 800 µL of DPPH 50 µM and then the solution was measured at 517nm every 5 min for 30 min. Absorbance against time was plotted (n=6). On the other hand, different solutions were employed from working stock of 0.2 mg/mL in 3% of Tween 80. $IC_{50}$ was calculated using straight line equation.

Digital analysis of chromatographic plates

Analysis regions were established to measure optical intensities, these regions were analyzing through ImageJ 1.52 a (National Institute of Health, USA) software.[9]

Statistical analysis

All experiments were conducted in triplicate and analyzed statically with one-way ANOVA, means values were subjected to the Tukey test using Minitab® 17.1.0 software.

## RESULTS

Efficiency of maceration and UPAE extraction methods

The percentage yield of UPAE and macerating are show in figure 1a, the percent yield of UPAE is the average of all sonication times. Compared to maceration, UPAE was more efficient in both, leaf and bark. The extract yield recovered, was 290% in leaves and 270% in bark. Figure 1 b indicates that after 5 min the maximum yields of leaves extraction were reached by UPAE method, so that in later times it did not significantly affected obtaining the extracted solids.

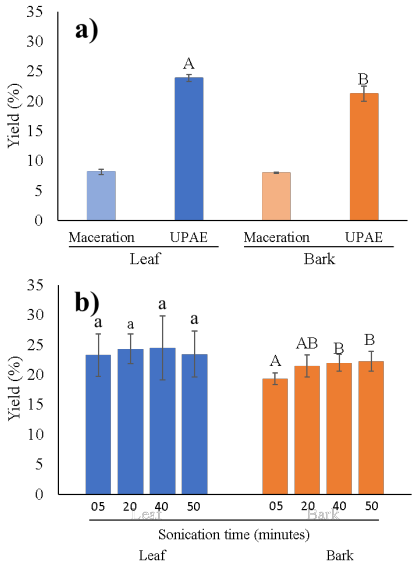


**Figure 1. Extraction efficiency employing ultrasonic and pressing assistance or maceration.** a) Difference in yield employing maceration or UPAE method is showed. b) Effect of different times of sonication on solids extracted yield with UPAE is showed. Different letters, indicate significative differences ($p \leq 0.05$) (Capital letters for leaf, lower case for bark).

In bark, the higher UPAE yield was obtained at 20 min when the significant differences ($p \leq 0.05$) were calculated by LSD statistical method, but when this was calculated by Tukey statistical method not statistical difference were found (Figure 1 b). it was showed that the yield in leaves is higher than bark at the 5 and 20 min of UPAE, 40 and 50 min are not statistically different.

Antioxidant activity

Semiquantitative preliminary antioxidant activity determination, in TLC plates, showed that solids extracted in different ultrasonic times exhibit antioxidant capacity similar in both, leaves and bark (Figure 2).

TLC plates analysis by densitometry, showed that 20 µg extracts has antioxidant activity apparent, equivalent to 1.66-1.81 and 1.45-1.56 µg of ascorbic acid, in bark and leaves, respectively

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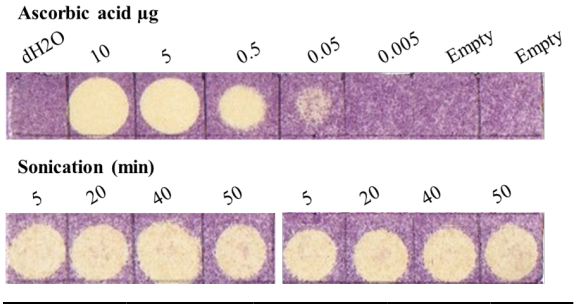


| | Leaf | | Bark | |
|---|---|---|---|---|
| t sonication (min) | Relative value to ascorbic acid (µg) | | | |
| | Mean | SD | Mean | SD |
| **5** | 1.78 * | ± 0.31 | 1.56 † | ± 0.22 |
| **20** | 1.68 * | ± 0.17 | 1.56 † | ± 0.21 |
| **40** | 1.81 * | ± 0.19 | 1.53 † | ± 0.16 |
| **50** | 1.66 * | ± 0.19 | 1.45 † | ± 0.13 |

**Figure 2. Semiquantitative antioxidant activity preliminary determination (densitometry).** Different symbols indicate significative differences ($p \leq 0.05$) (* between time in leaf and † between time in bark).

The antioxidant capacity from extracts obtained by different sonication times, is show in figure 3. The scavenging of DPPH radical is the same, independently of the sonication time. On the other hand, the antioxidant activity of Tween 80 was tested because it was employed as dissociating agent in this work, in both, leaves and bark. Then, was determinate that tween has not effect on the scavenging of DPPH radical.

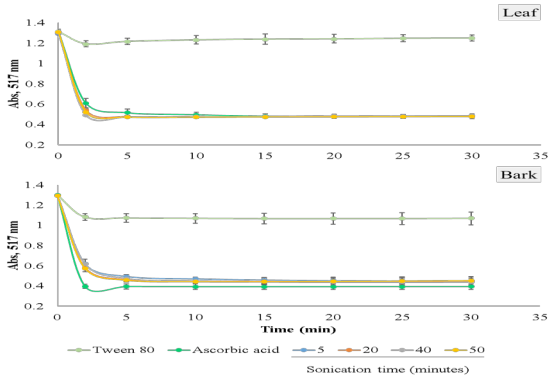


**Figure 3. Qualitative spectrophotometric method to determination of antioxidant activity in ethanolic extracts UPAE.** Antioxidant activity between different sonication times extracts was tested, using ascorbic acid as positive control (Ascorbic acid). Additionally, antioxidant activity of Tween 80 was discarded.

The inhibitory concentration to scavenging of half DPPH radical ($IC_{50}$), was tested for spectrophotometric method as mentioned previously and with the absorbance values was calculated the $IC_{50}$ parameter for each sonication time, in both leaf and bark. No significant differences ($p \leq 0.05$) were found between sonication times, for this reason is considered that since from 5 min of sonication is enough to extract entire activity in both leaves and bark. Results are in the table 1.

**Table 1.** Inhibitory concentration to scavenging of half DPPH radical ($IC_{50}$) of leave or bark extracts with different sonication time.

| Sonication | $IC_{50}$ (mg/mL) | | | |
|---|---|---|---|---|
| (min) | LEAF | | BARK | |
| | MEANS | SD | MEANS | SD |
| **5** | 0.00827 * | ± 0.001 | 0.01154 † | ± 0.003 |
| **20** | 0.00763 * | ± 0.001 | 0.01094 † | ± 0.003 |
| **40** | 0.00871 * | ± 0.001 | 0.01064 † | ± 0.003 |
| **50** | 0.00872 * | ± 0.001 | 0.01348 † | ± 0.002 |

* or † indicate significant differences ($p \leq 0.05$).

However, results were significantly higher in $IC_{50}$ values for the bark. The leaf is more potent since it requires less concentration of the extract to reduce 50% of the DPPH radical.

The ethanolic extracts of leaf and bark were separated trough TLC test with a mobile phase previously mentioned. It was found not difference between patrons of antioxidant compounds distribution when were revelated with DPPH solution (0.15%). Additionally, it was observed that some compounds were maintained at the point of application, while others were distributed along of TLC plate due that the mobile phase was polar, the compounds had not carried out were considered as no polar, while the compounds distributes along the plate were considered as polar. Accordingly, we can say that the extract has polar and no polar antioxidant compounds, so that the differences

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of the non-polar compounds observed in the plates of the different times of sonication of the bark seem not to affect to the total antioxidant activity. A representative image is show in figure 4, which showed general distribution of total compounds and the regions were the antioxidant activity was located.

On other hand, the areas with compounds in the starter line were considered as no polar and those carried out for the mobile phase, were considered as polar compounds.

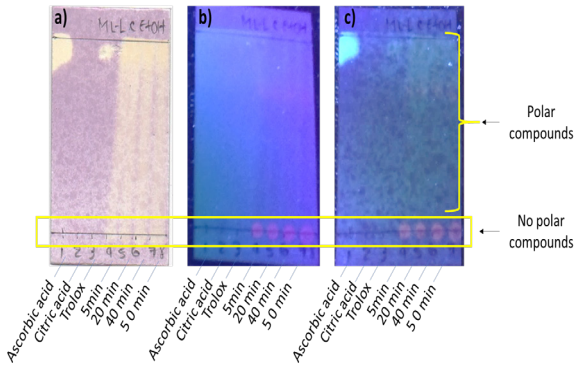


**Figure 4. Representative images of the TLC plates of compounds with antioxidant activity in bark ethanolic extract revealed with: a) DPPH, b) long wave (365 nm) and c) DPPH + long wave (365 nm).** The compounds attached at the start line were considered non-polar, the compounds entrained by the mobile phase were considered polar.

Additionally, some regions that fluoresce at 365 nm and after treatment with DPPH lost fluorescence, were considered as compounds with antioxidant activity. Regions of analysis were selected with ImageJ, as previously described. Table 2 shows results of digital analysis for regions (positive at 365 nm and DPPH).

The antioxidant activity and total polar compounds are similar ($p \leq 0.05$) for all sonication times, in both leaves and bark. However, the no polar compounds of the bark decreased significant at 50 min.

**Table 2.** Evaluation of the significant differences ($p \leq 0.05$) in TLC plates of different extracts using Image J.

| SONICATION (MIN) | POLAR COMPOUNDS | | | | | |
|---|---|---|---|---|---|---|
| | LEAF | | | BARK | | |
| | DPPH | λ | λ + DPPH | DPPH | λ | λ + DPPH |
| 5 | A | A | A | A | A | A |
| 20 | A | A | A | A | A | A |
| 40 | A | A | A | A | A | A |
| 50 | A | A | A | A | A | A |
| | NO POLAR COMPOUNDS | | | | | |
| | LEAF | | | BARK | | |
| | DPPH | λ | λ + DPPH | DPPH | λ | λ + DPPH |
| 5 | A | A | A | A | B | A |
| 20 | A | A | A | A | AB | A |
| 40 | A | A | A | A | AB | A |
| 50 | A | A | A | A | A | A |

Different letter is significant different ($p \leq 5$). λ indicate 365 nm.

## DISCUSSION

The ultrasonic assisted extraction is a widely method used in laboratory processes for extraction,[5,10,11,12,13] however, there are not consensus regarding the effect of sonication time on obtaining compounds.[11,13]

Results shown that prolonged times of sonication (more than 5 min in leaves and more than 20 min in bark by Tukey, $p \leq 0.05$ ) not contribute significantly to the recovery yields of total solids or total compounds with antioxidant activity, which is similar finding to that reported by Al-Juhaimi *et al.* (2016).[14]

In this work, it was observed a total yield of solids in leaves of 23.87% ± 0.61, this is equivalent at increased 290% with respect to leaf extracts obtained by conventional maceration. In the bark, the yield obtained was 21.23% ±1.30, this result is equivalent to an increased 270% with respect to bark extracts obtained by conventional maceration. Is important to remark that we use only 4 g of flour. The yields obtained by UPAE, are higher at

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the reported by other authors in extractions assisted by sonication.[10,11,12,13] It remains to be determined which factor, sonication, pressing o a combination, have a key role in the recovery; as is known that the filtration separation method is inefficient although is widely used, either by gravity or assisted by vacuum.

The aim of this work was extract at room temperature and a single extraction cycle the antioxidant compounds trough ultrasonic system and pressing, although Lopez *et al.,* (2016)[10] report the use of ultrasonic assistance extraction trough the increases of temperature (60 °C), which according to the authors could improve the yield. de Souza *et al*., (2017)[15] performance the extraction of polyphenols using a temperature of 60 °C and increasing the number of re-extractions. Therefore, it is not ruled out the possibility of changing these, temperature and many extractions cycles in future works, because the information about the effect of temperature or numbers of extractions cycles is poor.

Finally, it is important to note, that the sonication time in the UPAE does not significantly alter the total antioxidant activity, not by regions and not the potency of the extracts. So, in the case of B. *crassifolia*, it is suggested that for leave 5 min of UPAE is enough and for bark a maximum of 20 min are required to obtain a higher yield of solids and compounds with antioxidant activity.

## CONCLUSIONS

This study demonstrated that the ultrasonic assistance extraction and the separation method by pressing, improves solids yields and compounds obtained without affecting the antioxidant activity, as demonstrated in the preliminary analysis in TLC and the spectrophotometric determination of the $IC_{50}$. With this method is possible to perform extractions to quantities as small as 4 g of flour used in this study. So, it would be advisable in studies when the material is limited.

Finally, without making a specific identification, this study shown that *B. crassifolia* produces a great variety of antioxidant compounds in both leaf and bark of polar and apolar type. So, it is important since it has an efficient extraction method, the compounds obtained and their possible applications must be studied.

It is important to highlight that in this work the use of ultrasound in *B. crassifolia* is reported for the first time.

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