

High-Pressure Fractionation of Tropical Fruits with Potential Antibacterial Activity: *M. Indica* L. and *B. Guineensis*

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The great interest in the potential health benefits of tropical fruits is due to their high content of antioxidants and phytochemicals. Colombia ranks as the second country with the major biodiversity worldwide. *B. guineensis* (Arecaceae) is a palm that grows in Colombia and Central America. The purple-black fruits of this plant are rich in thermal-stable anthocyanins. *M. indica* L. (Anacardiaceae) is a great source of phenolic compounds. It has multiple functional properties including antioxidant, antimicrobial, antidiabetic and anticarcinogenic activities. In this work, high-pressure extraction techniques: supercritical fluid extraction (SFE) and enhanced solvent extraction (ESE), and two different fractionation techniques: *i*) cascade fractionation and *ii*) sequential fractionation were applied. Fractions were analyzed by means of their phenolic content, antioxidant activity, and antibacterial activity against different bacterial strains: *E. coli*, *P. mirabilis*, *S. Aureus*, *S. enteritidis*, *E. aerogenes* and *P. aeruginosa*. The sequential fractionation of *B. guineensis* pulp consisted in three steps: 1) supercritical CO₂, 2) CO₂ + 50% ethanol, and 3) CO₂/EtOH/H₂O (50:25:25). A red fraction rich in phenolic compounds, high antioxidant and antibacterial capacity (inhibition zone ~ 10 mm) was obtained in the last step. A cascade fractionation of *M. indica* leaves using CO₂ + 50% H₂O and three separators (S1, S2 and S3) was evaluated. Fractions obtained in S1 and S2 presented antioxidant capacity and antibacterial activity against *P. mirabilis*, and S2 also against *S. Aureus* and *Salmonella*.

1. Introduction

Colombia is a privileged country with a great diversity of ecosystems and climates attributed to its equatorial geography and its complex topography (Bernal et al., 2011; Bernal et al., 2006). *B. guineensis*, commonly known as corozo, corozo de lata or coyol, is native to Central and South America including the Colombian Caribbean coast. The red to violet-black color fruit is rich in anthocyanins such as cyanidin-3-rutinoside and cyanidin-3-glucoside (87.9%) (Osorio et al., 2011). *B. guineensis* extracts have shown antioxidant and cytoprotective activity (Osorio et al., 2011; Rojano et al., 2012). *M. indica* is another tropical fruit rich in phenolic compounds with numerous pharmaceutical properties including antioxidant, antimicrobial, antidiabetic and anticarcinogenic activity (Fernández-Ponce et al., 2012). Different innovative techniques have been explored to obtain antioxidant extracts from *M. indica* leaves including supercritical fluid extraction (SFE) and pressurized liquid extraction (PLE) which are recognized as green extraction techniques due to they employ Generally Recognized as Safe (GRAS) solvents (CO₂, water or ethanol). *M. indica* leaf extracts with potent antioxidant activity (Fernández-Ponce et al., 2013; Fernández-Ponce et al., 2015) and potential applications in the treatment of diabetes (Infanta-García et al., 2017), cancer (Fernández-Ponce et al., 2017) and neurodegenerative diseases (Infanta-García et al., 2017a; Infanta-García et al., 2017b) have been obtained by such techniques. The use of CO₂ as solvent provides many advantages including the reduction of liquid solvent consumption and extraction time, the avoidance of sensitive compounds degradation and the enhancement of mass transfer phenomena thus increasing the yield of the process (Casas et al., 2009; Fernández-Ponce et al., 2012). In addition, it is possible to design fractionation processes such as: *i*) the

cascade fractionation that employs multiple cyclonic separators connected in series where it is possible to collect fractions of different chemical compositions by changing CO₂ solvent capacity with small modifications of pressure and temperature (Fuentes-Gandara et al., 2019), or *ii*) sequential extraction with CO₂ and increasing the percent of polar cosolvents such as ethanol or water (Paula et al., 2014).

Having into account the great potential of *M. indica* leaf and *B. guineensis* pulp extracts as antioxidant agents in food preservation or other nutraceutical or pharmaceutical applications, the aim of the present work was focused on the evaluation of different high-pressure fractionation techniques in order to obtain fractions with antioxidant and antimicrobial activities. The different fractions were also analyzed in terms of their chemical composition.

2. Material and Methods

2.1 Raw materials and reagents

M. indica cv. Kent leaves were provided by The Institute for Mediterranean and Subtropical Horticulture 'La Mayora', Superior Centre of Scientific Researches (CSIC), Malaga, Spain. Leaves were collected in March 2016. *B. guineensis* fruits were collected in Barranquilla, Colombia in April 2016. Raw materials were dried in an oven at 70 °C until constant weight, grounded and kept in absence of light. Carbon dioxide (99.995%) was provided by Abello-Linde S.A. (Barcelona, Spain). Ethanol and acetonitrile (HPLC grade) were supplied by Panreac (Barcelona, Spain). 2,3-diphenyl-1-picrylhydrazyl (DPPH), gallic acid, peptone, tryptic soy agar (TSA) and breath heart infusion broth (BHI) were provided by Sigma-Aldrich (Steinheim, Germany). The Sensi-Discs for antimicrobial susceptibility test of Sulpha/trimethoprim (COT) and ceftriaxone (CTR) were purchased from BD BBL (United States) and amikacin (AK) from OXOID Thermo Fisher Scientific Inc. (United Kingdom). Milli-Q water was obtained from a millipore equipment (MilliQ®, Germany).

2.2 High-pressure equipment at pilot-plant scale

Extraction and fractionation tests were carried out in a high-pressure pilot plant supplied by Thar Technology (Pittsburgh, PA, USA, model SF5000). The equipment comprises two extraction vessels (5 L capacity) provided with a thermostatic jacket and a cartridge to load the sample, two pumps high-pressure pumps (for CO₂ and cosolvent, a heat exchanger, a back-pressure regulator valve, three cyclonic separators (500 mL each), a cooler and a liquid CO₂ storage tank. A schematic diagram of the pilot plant is shown in Figure 1.

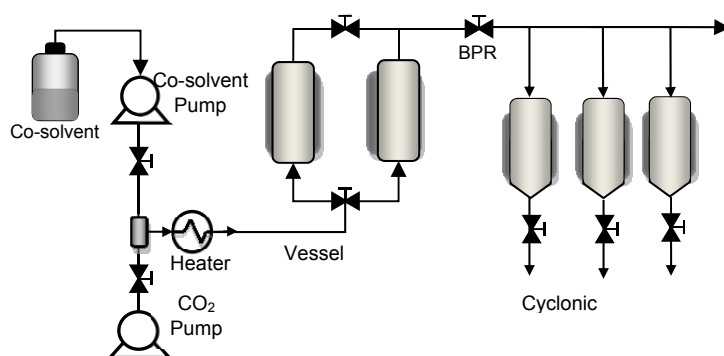


Figure 1: Schematic diagram of the high-pressure equipment at pilot-plant scale

Different fractionations techniques were necessary to be carried out according to the chemical composition of raw material. *B. guineensis* is rich in anthocyanins and phenolic compounds, and sequential fractionation has demonstrated to be successful to fraction anthocyanins from different raw materials (Seabra et al., 2010). On the contrary, a cascade fractionation by modifying pressure and temperature in different separators could result more favorable for *M. indica* leaf extracts due to they are rich in phenolic compounds.

2.3 Extraction and cascade fractionation of *M. indica* leaf

M. indica leaves (200 g) were extracted with a mixture of CO₂ + 50% H₂O at 300 bar of pressure and 70 °C of temperature. Fractionation conditions in the three separators were as follows: Separator 1 (S1): 200 bar/60 °C; Separator (S2): 100 bar/45 °C; Separator 3 (S3): 1 atm/30 °C. The solvent flow rate was maintained at 20 g/min for 5 h. After extraction time, the separators were depressurized and the fractions were collected in dark flasks and conserved at 4 °C for further analysis.

2.4 Extraction and sequential fractionation of *B. guineensis* pulp

B. guineensis pulp (200 g) was first extracted with pure CO₂ at 400 bar of pressure and 55 °C of temperature, and using a flow rate of 20 g/min during 3h. A subsequent extraction was applied with a mixture of CO₂ + 50% ethanol at 200 bar, 110 °C and 10 g/min for 3h. Finally, a third consecutive extraction was carried out with a mixture of CO₂/EtOH/H₂O 50:25:25 at 200 bar, 110 °C and 10 g/min for 3h. The different fractions were collected in dark flasks and conserved at 4 °C for further analysis.

2.5 Chemical composition of the extracts

The chemical compositions of the extracts were analyzed using an analytical HPLC series 1100 system (Agilent, Germany). The HPLC equipment comprises a degasser, a quaternary pump, an autosampler, a Synergi Hydro–RP C18 column (150 mm × 3 mm i.d., 4 μm) with a 4.0 mm × 2.0 mm i.d. C18 ODS guard column and a UV/vis detector (Phenomenex, USA), and a ChemStation® HP software. The elution method was described in previous studies (Fernández-Ponce et al., 2015). Total phenolic content was calculated as the sum of the peak areas quantified at 278 nm. Results were determined according to the calibration curve for gallic acid from Eq(1) and expressed in terms of mg of gallic acid equivalent (GAE)/100 g dried raw material.

$$A_{\text{gallic acid}} = 75.056 \cdot C + 411.11 \quad (1)$$

2.6 Antioxidant activity by DPPH assay

The antioxidant activity of extracts was determined by the DPPH assay. Different concentrations of extract were tested (0–2000 ppm). For each concentration, extract solution in ethanol (0.1 mL) was added to a 6 × 10⁻⁵ mol/L ethanol DPPH solution (3.9 mL). The decrease in absorbance was determined at 515 nm at different times until the reaction had 'reached a plateau'. The exact initial DPPH concentration (C_{DPPH}) in the reaction medium was calculated according to the calibration curve (r = 0.9999) shown in Eq(2):

$$Abs_{515 \text{ nm}} = -0.0065 \cdot 29.3112(C_{\text{DPPH}}) \quad (2)$$

A plot of % remaining DPPH vs. antioxidant concentration was generated. Antiradical activity was defined as the amount of antioxidant required to decrease the initial DPPH concentration by 50% [Efficient Concentration = EC₅₀ (mg extract/mg DPPH)]. Data were expressed as the antioxidant activity index (AAI), calculated in terms of 1/EC₅₀. The experiments were carried out in triplicate. AAI < 1.0 corresponds to a low AA, ≥ 1.0 corresponds to a good AA, and ≥ 2 represents very potent activity (Scherer & Godoy, 2009).

2.7 Antibacterial activity by disk diffusion susceptibility

The standardized bacterial inoculum of *Escherichia coli* ATCC 25922, *Salmonella enteritidis* ATCC 13076, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *Proteus mirabilis* ATCC 12453 and *Enterobacter aerogenes* ATCC 13048 by viable cell counting method at 10⁶ CFU/mL in peptone water (1%) was spread over the surface of BHI agar plates. Stock solutions of extracts (100 μg/mL) were prepared in 1% DMSO peptone water. Sterilized disks (6 mm diameter) by U.V. light, containing 80 μL of extract stock solution, were placed over BHI agar surface. 1% DMSO peptone water was used as positive control, sulpha/trimethoprim (COT), ceftriaxone (CTR) and amikacin (AK) as negative controls. Agar plates were incubated at 37 °C for 24 h. Afterwards, the inhibition growth zone diameter of samples was measured.

3. Results and discussion

3.1 Cascade fractionation of *M. indica* leaf extract

A cascade fractionation using CO₂ + 50% H₂O as solvent system and three cyclonic separators connected in series was applied for *M. indica* leaf extract. Data for the global yield, total phenolic content (TPC) and antioxidant activity (AA) were shown in Table 1.

Table 1: Global yield, total phenolic content and antioxidant activity of *M. indica* leaf fractions

<i>M. indica</i> leaf fractions	Global yield (%)	TPC (mg GAE/g)	AAI (μg DPPH/μg extract)
S1: 200 bar, 60 °	10.60 ± 1.12	309.17 ± 7.97	3.37 ± 0.09
S2: 100 bar, 45 °C	3.86 ± 0.12	259.17 ± 5.13	2.82 ± 0.04
S3: 1 atm, 30 °C	0.18 ± 0.03	26.52 ± 0.97	0.31 ± 0.02

*TPC: total phenolic content, AAI: antioxidant activity index, GAE: gallic acid equivalent.

Overall extraction yields and phenolic content decreases as the pressure of the separator decreases (Table 1). When a high proportion of a polar solvent is added to the CO₂ phase, a change in the polarity of the solvent occurs, which in turns allows a greater yield of polar substances such as polyphenols. When adding a compressible gas (more often CO₂) liquids can be divided in three classes, depending on the ability to dissolve CO₂. Water corresponds to *Class 1* which has insufficient ability to dissolve CO₂ and two liquid phases in equilibrium are formed (Fuentes-Gandara et al., 2019). In this case, two liquid fractions rich in water were collected in the first (S1) and second (S2) separator, being S1 that with the largest amount of extract. These fractions also presented high content of phenolic compounds and potent antioxidant activities (~3.0 µg DPPH/µg extract). Previous studies have also shown potent antioxidant activity for *M. indica* leaf extracts (3.55–5.64 µg DPPH/µg extract) (Fernández-Ponce et al., 2015). A solvent free fraction (S3), by contrast, was obtained in the third separator due to the low capacity of water to expand in the CO₂. Similar results were observed in previous studies where the cascade fractionation of sunflower leaf extracts using CO₂/H₂O solvent mixtures was explored (Fuentes-Gandara et al., 2019). In the last separator must be essentially collected compounds that remain soluble in pure CO₂, such as volatile and low polar compounds. This fraction presented a poor antioxidant activity due to the low content of phenolic compounds.

3.2 Sequential fractionation from *B. guineensis* pulp

Sequential fractionation of *B. guineensis* pulp was carried out by using first pure CO₂ as solvent, secondly it was applied an extraction with CO₂ + 50% EtOH, and a third consecutive extraction with a mixture of CO₂/H₂O/EtOH 50:25:25. Data about the global extraction yield, phenolic content and antioxidant activity of the fractions obtained are shown in Table 2.

Table 2: Global yield, total phenolic content and antioxidant activity of *B. guineensis* fractions

<i>B. guineensis</i> leaf fractions	Global yield (%)	TPC (mg GAE/g)	AAI (µg DPPH/µg extract)
F1: Pure CO ₂ (400bar, 55 °C)	0.31 ± 0.14	--	--
F2: CO ₂ + 50% EtOH (200 bar, 110 °C)	0.57 ± 0.18	58.13 ± 8.85	0.11 ± 0.01
F3: CO ₂ /EtOH/H ₂ O (200 bar, 110 °C)	2.29 ± 0.04	329.88 ± 9.43	1.53 ± 0.12

*TPC: total phenolic content, AAI: antioxidant activity index, GAE: gallic acid equivalent.

Yields obtained with pure CO₂ and CO₂ + 50% EtOH were too low. Phenolic compounds were not identified in F1 and it presented a very poor antioxidant capacity (AAI<1.0). A yellow fraction F2 was obtained with CO₂ + 50% EtOH, and some quantity of phenolic compounds was possible to be recovered (58.13 mg GAE/g dried extract). However, the increase of the solvent polarity by adding water to the solvent system (CO₂/EtOH/H₂O) enhanced the global yield (~2.0 %), phenolic content (329.9 mg GAE/g dried extract) and antioxidant activity (AA>1.0), besides a red fraction (F3) was obtained which indicated the possible presence of anthocyanins. Paula et al. also observed the influence of polarity on the overall extraction yields of fractions from *Baccharis dracunculifolia* obtained by sequential extraction with CO₂ – ethanol – water (Paula et al., 2017). Previous studies have also reported the obtaining of yellow fractions from elderberry pomace with CO₂/EtOH (10–50%), and anthocyanin-rich fractions were recovered only when the percent of ethanol was above 80% or water was added to the solvent system (Seabra et al., 2010). The efficiency of subcritical mixtures is attributed to the acidity drop of CO₂/water or CO₂/alcohol mixtures by the generation of carbonic and alkyl carbonic acid. This temporary pH drop leads to higher diffusivities by the increment of cell membrane permeability and brings stability to unstable molecules such as anthocyanins (Paula et al., 2017). The sequential fractionation is an advantageous technique due to nonpolar and low polar compounds can be removed in a first extraction with pure CO₂ and, thus, follow fractions more concentrated in polar compounds, such as polyphenols, can be obtained by the addition of ethanol and/or water to the solvent system (Paula et al., 2017; Seabra et al., 2010).

3.3. Antibacterial activity of *M. indica* leaf fractions

Data of antibacterial activity of *M. indica* fractions are shown in Table 3. The cascade fractionation of *M. indica* leaf extracts led to obtain two active fractions in the first (S1) and second separator (S2), whereas the fraction obtained in the third separator (S3) did not show antibacterial activity. Fractions presented antibacterial activity against all bacteria studied, except for *E. aerogenes*. A genetic study has shown that this bacterium have resistant genes to different compounds (Moura et al., 2017). On the other hand, S2 showed a higher inhibition zone for *E. coli* and *P. aeruginosa*; similar findings were shown by Singh et al. in 2015 from *M. indica* steam bark extracts (Singh et al., 2015). Not difference among S1 and S2 were observed for the results obtained with *S. aureus* and *S. enteritidis*. And a higher antibacterial susceptibility was observed for S1 with *P. mirabilis*, in fact, higher than that reported by Singh et al (2015).

Table 3: Antibacterial activity of *M. indica* leaf fractions

Fraction	Bacterial strain						
	<i>E. coli</i>	<i>P. mirabilis</i>	<i>S. aureus</i>	<i>S. enteritidis</i>	<i>E. aerogenes</i>	<i>P. aeruginosa</i>	
<i>M. indica</i> leaf	S1	8,30 ± 0,18	11,09 ± 0,74	10,38 ± 0,23	11,63 ± 0,21	--	9,60 ± 0,36
	S2	12,06 ± 0,11	8,97 ± 0,36	9,53 ± 0,21	10,38 ± 0,38	--	13,24 ± 0,23
	S3	--	--	--	--	--	--
Control	COT	26.75 ± 0.20	30.40 ± 0.29	29.24 ± 0.15	31.58 ± 0.49	25.43 ± 0.22	--
	CTR	31.04 ± 0.35	43.84 ± 0.19	28.29 ± 0.13	29.05 ± 0.22	27.23 ± 0.33	21.31 ± 0.34
	AK	19.36 ± 0.33	24.28 ± 0.50	26.65 ± 0.17	22.32 ± 0.28	23.13 ± 0.34	22.21 ± 0.15

*Negative controls (-): Sulpha/trimethoprim (COT), ceftriaxone (CTR) and amikacin (AK)

3.4. Antibacterial activity of *B. guineensis* pulp fractions

Data for antibacterial activity of *B. guineensis* fractions are shown in Table 4. As far as *B. guineensis* pulp is concerned, it was observed that the first fractions collected, F1 and F2, which were obtained with pure CO₂ and CO₂ + 50% EtOH, did not show antibacterial activity. These fractions presented poor phenolic content. Fraction F3, by contrast, shown antibacterial susceptibility against all the strain evaluated with inhibition zones of around 10 mm of diameter. The highest inhibitions of bacterial growth were observed for *E. aerogenes*, *S. enteritidis*, and *P. aeruginosa*. In the case of *E. aerogenes* the diameter obtained was of 12 mm; this result may be promising because few studies have been reported using this type of compounds against this bacterium. The fraction F3 of *B. guineensis* pulp presented phenolic compounds but also must contain anthocyanins which could enhance the antibacterial activity against the bacteria analyzed, as has been shown in other works (Leyva-Jimenez et al., 2018; Ng et al., 2018).

Table 4: Antibacterial activity of *B. guineensis* pulp fractions

Fraction	Bacterial strain						
	<i>E. coli</i>	<i>P. mirabilis</i>	<i>S. aureus</i>	<i>S. enteritidis</i>	<i>E. aerogenes</i>	<i>P. aeruginosa</i>	
<i>B. guineensis</i>	F1	--	--	--	--	--	--
	F2	--	--	--	--	--	--
	F3	10.27 ± 0.80	10.93 ± 0.30	8.49 ± 0.06	11.15 ± 0.40	12.91 ± 0.19	11.61 ± 0.21
Control	COT	26.75 ± 0.20	30.40 ± 0.29	29.24 ± 0.15	31.58 ± 0.49	25.43 ± 0.22	--
	CTR	31.04 ± 0.35	43.84 ± 0.19	28.29 ± 0.13	29.05 ± 0.22	27.23 ± 0.33	21.31 ± 0.34
	AK	19.36 ± 0.33	24.28 ± 0.50	26.65 ± 0.17	22.32 ± 0.28	23.13 ± 0.34	22.21 ± 0.15

*Negative controls (-): Sulpha/trimethoprim (COT), ceftriaxone (CTR) and amikacin (AK)

4. Conclusions

The results obtained in this study showed high-pressure fractionation techniques, such as cascade fractionation and sequential extraction fractionation, are efficient techniques to fractionate plant extracts from tropical species such as *M. indica* L. and *B. guineensis*. Two *M. indica* leaf active fractions were obtained in the first (S1) and second separator (S2) when a cascade fractionation with three cyclonic separators was applied. These fractions presented high content of phenolic compounds which contributed to their antioxidant and antibacterial activity against foodborne pathogens such as *E. coli*, *S. enteritidis*, *S. aureus*, *P. aeruginosa*, and *P. mirabilis*. On the other hand, the fractionation of *B. guineensis* pulp by sequential extraction, first with pure CO₂, secondly with CO₂ + 50% EtOH and finally with CO₂/EtOH/H₂O 50:25:25, led to obtain a third red fraction (F3) rich in phenolic compounds and anthocyanins and with good antioxidant and antibacterial activity against all bacteria studied including also *E. aerogenes*. This preliminary study shown the potential use of cascade or sequential high-pressure fractionation to obtain active fractions from *M. indica* leaves and *B. guineensis* pulp with potential applications in food preservation by avoiding oxidation or delaying the growth of foodborne pathogens. However, further studies are necessary to optimize these fractionation methods in order to increase the richness in active compounds and functional activity of plant extract fractions.

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