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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 antioxidant minerals 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, the use of 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. Fractions were analyzed by means of their phenolic content, antioxidant activity, and antibacterial activity against different bacterial strains: *E. coli, P. mirabilis, S. Aurerus, 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 obtained 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-Garcia et al.b, 2017), cancer (Fernández-Ponce et al., 2017) and neurodegenerative diseases (Infanta-Garcia 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 o 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**.

Co-solvent

Heater

Cyclonic Separators

CO2 Pump

Co-solventPump

Vessel Extractors

BPR

*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 due to yellow fractions are obtained with pure CO2 or mixtures CO2 + ethanol whereas red fractions rich in anthocyanins are only possible to be obtained after the addition of water to the solvent system (Seabra et al., 2010). On the contrary, the sequential fractionation is not so successful for *M. indica* leaf extracts due to this raw material is rich in phenolic compounds. A cascade fractionation by modifying pressure and temperature in different separators could result more favorable for this raw material.

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.

|  |  |
| --- | --- |
|  | (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 (CDPPH) in the reaction medium was calculated according to the calibration curve (r = 0.9999) shown in Eq(2):

|  |  |
| --- | --- |
| ) | (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* ATCC12453 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 fractionation of *M. indica* leaf extract was carried out by applying a cascade fractionation using three cyclonic separators connected in series. The three fractions were evaluated in terms of the global yield, total phenolic content (TPC) and antioxidant activity (AA). Data were shown in **Table 1**.

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

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ***M. indica* leaf**  **fractions** | **Global yield (%)** | **TPC**  **(mg GAE/g)** |  | **AAI**  **(µg DPPH/µg extract)** |
| S1: 200 bar, 60 º | 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.

Mixtures of CO2 + 50% cosolvent are considered as subcritical mixtures due to the addition of large proportions of water or polar organic solvents to CO2 (>10–20 mol %) increases the critical temperature (*Tc*) of the resulting mixture and, thus, leading to extractions below the critical point. These mixtures are also called as enhanced fluidity liquids. They combine the properties of liquids with the superior transporting properties of supercritical fluids. Mass transfer phenomena are enhanced by decreasing viscosity, reducing interfacial tension and increasing diffusivity due to the dissolution of CO2 in the liquid solvent. On the other hand, water swells the sample thus leading to an easier penetration of the solvent into the matrix and helps to disrupt matrix-analyte interactions which increases the extraction yield (Fernández-Ponce et al., 2015; Fuentes-Gandara et al., 2019).

From **Table 1**, it can be observed that the overall extraction yields and phenolic content decreases as the pressure of the separator decreases. 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 I* which has insufficient ability to dissolve CO2 and has no significant changes in their properties. In this sense, two liquid phases in equilibrium are formed with CO2/H2O mixture (Fuentes-Gandara et al., 2019). The formation of one or two phases affects the behaviour of the compressed mixture during the fractionation. 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 were 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 in order to increase the polarity of the solvent system and favor the obtaining fractions with different chemical composition. 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

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| ***B. guineensis* leaf**  **fractions** | **Global yield (%)** | **TPC**  **(mg GAE/g)** |  | **AAI**  **(µg DPPH/µg extract)** |
| F1: Pure CO2 (400bar, 55 ºC) | 0.31 ± 0.14 | -- |  | -- |
| F2: CO2 + 50% EtOH (200 bar, 110 ºC) | 0.57 ± 0.18 | 58.13 ± 8.85 |  | 0.11 ± 0.01 |
| F3: CO2/EtOH/H2O (200 bar, 110 ºC) | 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. Low polar compounds such as waxes and other volatiles compounds must be extracted with pure CO2. In fact, phenolic compounds were not identified in this fraction (F1) and it presented a very poor antioxidant capacity (AAI<1.0). The addition of ethanol as cosolvent increased the yield. 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, when water was added to the solvent system (CO2/EtOH/H2O 50:25:25) a substantial increase of the global yield (~2.0 %) and phenolic content (329.9 mg GAE/g dried extract) was observed. The increase of the solvent polarity by adding water to the solvent system enhanced phenolic extraction. Paula et al. also observed the influence of polarity on the overall extraction yields of fractions from *Baccharis dracunculifolia* obtained by sequential extraction with SC-CO2 – ethanol – water (Paula et al., 2017). In addition, a red fraction (F3) was obtained which indicated the presence of anthocyanins. 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, respectively. This temporary pH drop of the solvent system leads to higher diffusivities by the increment of cell membrane permeability. Moreover, it brings stability to unstable molecules such as non-acylated anthocyanins. This phenomena is advantageous due to it is possible to avoid the addition of organic acids necessary to enhance the extraction of anthocyanins and thus reducing environmental impact and complying health restrictions (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). Fraction F3 presented a high content of phenolic compounds. Moreover, **Table 2** shown that the antioxidant activity was also enhanced in the third extraction. A fraction F3 with good AA was obtained (>1.0). This antioxidant activity could be attributed to phenolic compounds but also to anthocyanins which are also considered as potent antioxidant compounds.

3.2 Antibacterial activity of fractions of *M. indica* leaf and *B. guineensis* fractions

According to inoculum standardization, the number of UFC/mL in 106 dilution used to each bacteria was 467 ± 48.1 UFC/mL of *E. coli*, 390 ± 49.9 UFC/mL of *P. mirabilis,* 143 ± 62.9 of *S. aureus,* 284 ± 7.1 UFC/mL of *S. enteritidis,* 730 ± 56.6 UFC of *E. aerogenes* and 323 ± 18.4 UFC/mL of *P. aeroginose.* Data obtained for *M. indica* leaf and *B. guineensis* pulp fractions were shown in **Table 3 and 4**. Data were expressed in terms of the diameter of the inhibition zone.

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

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

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Fraction** | | **Bacterial strain** | | | | | |
| ***E. coli*** | ***P. mirabilis*** | ***S. aureus*** | ***S. enteritidis*** | ***E. aerogenes*** | ***P. aeroginosa*** |
| *M. indica* 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)

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*, which an inhibition zone was not observed, 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 with solvent extraction, who obtained zones of inhibition with a size similar for these microorganisms 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).

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

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

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Fraction** | | **Bacterial strain** | | | | | |
| ***E. coli*** | ***P. mirabilis*** | ***S. aureus*** | ***S. enteritidis*** | ***E. aerogenes*** | ***P. aeroginosa*** |
| *B. guineensis* | 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)

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 shown antibacterial activity. This 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 obtainded 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 show in other works (Leyva-Jimenez et al., 2018; Ng et al., 2018).

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. aeroginosa,* 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 bacteries studied including also *E. aerogenes*. This preliminary studied 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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**REVIEWER RESPONSES**

REVIEWER # 1

Paper # 66

Title HIGH-PRESSURE FRACTIONATION OF TROPICAL FRUITS WIT....

by:Fernandez Ponce M.T.,Soto Varela Z.,Castro Gil P.,

**1)Is the contribution original and up-to-date?: good**

**2)Has it engineering / scientific relevance?: good**

**3)Is the presentation clear with a good expression of English?: good**

**4)Are references written according to Harvard Style? Yes**

R/ Harvard style referencing was improved

**5)Is this paper referencing any Chemical Engineering Transactions article?: no**

R/ CET references were included

decision: accept after minor revisions

**Comments by reviewer**

I congratulate the authors of this work. The sections are correctly divided, the subject presented clearly and objectively.

I pointed out only a few typos that probably went unnoticed:

**6)On page 2, in the section 2.1, the expression 70°C should be replaced by 70 °C, with a space between the number and the symbol of degrees. Because the norm recommends that a space be left before the symbol of unity, after the numbers representing the value of reatness.**

R/ Thanks for the suggestion. 70ºC was corrected to 70 ºC. This suggestion was considered throughout the manuscript

**7)Still in this section there is a dot in the sentence ...)(Barcelona, Spain).high-pressure (...). Please remove it.**

R/ The word high-pressure was removed from the text

**8)In the same sentence the word broth (broth were provided by Sigma-Aldrich) should be replaced by both.**

R/ The sentence was correct as: breath heart infusion broth (BHI)to avoid any confusion

After corrections, the work is accepted for me.

REVIEWER # 2

Paper # 66

Title HIGH-PRESSURE FRACTIONATION OF TROPICAL FRUITS WIT....

by:Fernandez Ponce M.T.,Soto Varela Z.,Castro Gil P.,

**1)Is the contribution original and up-to-date?: sufficient**

**3)Has it engineering / scientific relevance?: sufficient**

**3)Is the presentation clear with a good expression of English?: sufficient**

**4)Are references written according to Harvard Style?:no**

R/ References were written according to Harvard Style

**5)Is this paper referencing any Chemical Engineering Transactions** **article?:no**

R/ CET references were included

Decision: accept after major revisions

**Comments by reviewer**

**6)In some cases they speak of the scientific name of the fruit and in others of the common, please choose which of the two to use.**

R/ Thanks for the suggestion authors used the scientific name throughout the text.

**7)Table 2 is from corozo results, but in the informations says mango, please correct this ambiguety.**

R/ Table 2 was corrected. Information was about *B. guineensis* (corozo)

**8)It is not clear if there are also changes in pressure in corozo extraction.**

R/ Pressure and temperature were included in Table 2 for corozo pulp extraction.

**9)The mango and corozo results aren’t comparable, because this contitions are differents.**

R/ Thanks for the suggestion. Data about the antibacterial activity of *M. indica* and *B. guineensis* fractions were put in different sections taking into account the suggestion of the reviewer. Besides, any comparison between *M. indica* and *B. guineensis* was avoided.

On the author hand, authors considered necessary to include a brief justification about the selection of different fractionation techniques for *M. indica* and *B. guineensis* in Section 2.2:

“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 due to yellow fractions are obtained with pure CO2 or mixtures CO2 + ethanol whereas red fractions rich in anthocyanins are only possible to be obtained after the addition of water to the solvent system [13-14]. On the contrary, the sequential fractionation is not so successful for *M. indica* leaf extracts due to this raw material is rich in phenolic compounds. A cascade fractionation by modifying pressure and temperature in different separators could result more favorable for this raw material”.

**10) I do not see comparisons of previous studies to verify if the values obtained are coherent or not.**

**R/** Authors added different comparisons of data with previous works. However, comparisons for *B. guineensis* specific data were not possible to be provided because this raw material has not been exhaustively studied. Previous works about supercritical extraction/fractionation or antibacterial activity for *B. guineensis* extracts have not been published.

The comparisons included were as follows:

**Section 3.1:** “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) [7]. 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 were the cascade fractionation of sunflower leaf extracts using CO2/H2O solvent mixtures was explored [11]”

**Section 3.2:** “The increase of the solvent polarity by adding water to the solvent system enhanced phenolic extraction. Paula et al. also observed the influence of polarity on the overall extraction yields of fractions from *Baccharis dracunculifolia* obtained by sequential extraction with SC-CO2 – ethanol – water [13]. In addition, a red fraction (F3) was obtained which indicated the presence of anthocyanins. 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 [14]”

**Section 3.2.1: “**Fractions presented antibacterial activity against all bacteria studied, except for *E. aerogenes*, which an inhibition zone was not observed, a genetic study has shown that this bacterium have resistant genes to different compounds [16]. 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 with solvent extraction, who obtained zones of inhibition with a size similar for these microorganisms from *M. indica* steam bark extracts [17]. 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 [17]”