

Four methods of extraction in leaves of *Byrsonima crassifolia* and their effects in gastrointestinal smooth muscle strips

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Abstract

Byrsonima crassifolia leaves were extracted via maceration (*BrMa*), percolation (*BrPer*), soxhlet (*BrSox*) and sonication (*BrSon*). Spectrophotometric estimation of chlorophylls and analysis of cumulative extraction allowed the characterization of the extraction process. Under these experimental conditions, the extraction efficiency for each method was greater than 99%. The best yield was recorded by Soxhlet (7.29±0.21%), the best extraction rate was by sonication (m : 0.032±0.004; r^2 : 0.96±0.02) and maceration showed less variations in estimation of chlorophyll *a* and *b*. On the other hand, pharmacological evaluation in a rat ileum strips allowed identify that *BrMa*, *BrPer*, *BrSox* and *BrSon* reduce the spontaneous contractions (E_{max} : 38.54±2.03, 28.9±1.6, 31.87±1.79, and 29.09±1.27%; EC_{50} : 11.6±2.38; 13.22±2.83; 39.74±2.64 and >50 µg/mL, respectively) of ileum strips in a concentration-dependent manner, however, the effects were significantly lower than papaverine (E_{max} : 59.32±2.57%; EC_{50} : 7.11±1.0 µg/mL). These results indicated that according to extraction method there are variations in the metabolic content as well as in the pharmacological actions induced by *B. crassifolia* extracts. Finally, there are a low correlation between extraction temperature ($r > 0.87$) and spasmolytic activity.

Palabras clave: *Byrsonima crassifolia*, maceration, chlorophyll, ileum strips

Introduction

Current scientific research has proven the effectiveness of various drugs obtained from natural sources, being in some cases leading products in the treatment of diseases with high prevalence, in this sense, natural products are an important source for the search of molecules bioactive and the development of phytomedicines (Newman and Cragg, 2007). In Mexico there are numerous species with medicinal uses, Anacardiaceae, Apocynaceae, Asteraceae, Burseraceae, Cactaceae families provide a large number of them (Monroy Ortiz and Monroy, 2004) and in several regions of the Yucatan Peninsula have a wide biological variability (Arce-Ibarra and Armijio-Canto, 2011). According to ethnomedical reports various wild plant species are used in traditional medicine (La Torre-Cuadros and Islebe, 2003).

Byrsonima crassifolia (L.) KUNT (1822), (*Chi, maya*-Yucatan; nance, Spanish (Malpighiaceae) is a perennial tree widely distributed in southeastern Mexico; in traditional medicine is used as astringent, antidiarrheal, dysentery and stomach pain (Kunth, 1976). Phytochemical studies have allowed the identification of secondary metabolites such as polyphenolic compounds, hydrolyzable tannins, flavanols, proanthocyanidins (Gordon *et al.*, 2011) and others. Important polyphenolic compounds derived from the bark of *B. crassifolia* are 5-O-galloylquinic acid, 3-O-galloylquinic acid 3,4-di-O-galloylquinic acid 3,5-di-O-galloylquinic acid 3,4,5-tri-O-galloylquinic acid, (+)-epicatechin-3-gallate, (+)-catechin and (+)-epicatechin (Maldini *et al.*, 2011). Studies on the fruit have allowed the identification of volatile compounds such as ethanol (28.3%), ethylhexanoate (25.2%), methyl butyrate (2.8%) and butanoic (5.1%) and hexanoic acids (5.1 %) and other in low abundance (Alves and Franco, 2003).

Pharmacological studies on *B. crassifolia* have identified antimicrobial (Martínez-Vázquez *et al.*, 1999) antiparasitic (Cáceres *et al.*, 1998), antimycotic (Caceres *et al.*, 1991), actions at CNS level (Cifuentes *et al.*, 2001), antidepressants (Herrera-Ruiz *et al.*, 2011), antihyperglycemic and antihyperlipidemic (Perez-Gutierrez *et al.*, 2010), anti-inflammatory (Maldini *et al.*, 2009) and spasmolytic (Béjar and Malone, 1993) properties. In fact, quercetin 3-O-xyloside (12mg/kg), rutin (4.4mg/kg), quercetin (1.4mg/kg) and hesperidin (0.7mg/kg) are proposed as secondary metabolites associated with antidepressant effects (Herrera-Ruiz *et al.*, 2011).

In this ethnomedical, phytochemical and pharmacological context, the present work analyze different extraction process of leaves of *B. crassifolia* through estimation of the chlorophyll content and yield as well as evaluation of the spasmolytic activity induced by extracts on spontaneous contraction of isolated segments of rat ileum.

Methodology

Plant material: Leaves of *B. crassifolia* were collected in the municipal market of Campeche, Campeche, México in January 2016 and dried at room temperature, ground (Ika Works®) and stored according to FHEUM recommendations. The collected specimens were identified by MSc. Pedro Zamora-Crecencio and deposited in the Herbarium of the Autonomous University of Campeche.

Extraction: From dry and ground material (1g) as well as the use of MeOH as solvent (20ml) three consecutive extractions were performed via maceration (t:27h, T:25°C), Percolation (Q:1ml/min), sonication (ColeParmer®, t:10min) and Soxhlet (T:55°C;7cycles). In order to document variations in metabolic content, the chlorophyll content was estimated using the methodology of Pearson & Strickland (Mara *et al.*, 1994) and Hager & Meyer-Bertenrath (Hager and Meyer-Bertenrath, 1966). As a control of chlorophylls extraction, Hansmann method (Hm, Vol: 3mL acetone at 90%; t:24h; T:5°C) was used (Cortés *et al.*, 2017).

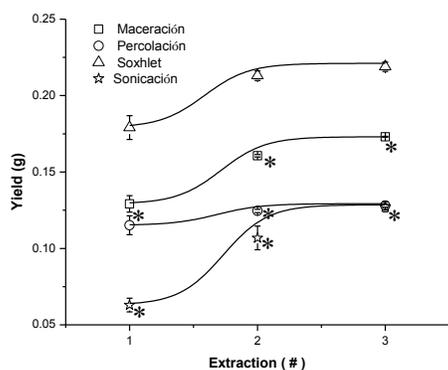
Animals: All experimental animals procedures used were reviewed and approved by the Animal Care and use Committee of University Autonomous of Campeche (No. PI-420/12). *Rattus norvegicus* (Wistar strain, male, 255±21 g) were maintained in a cycle of 12h light/dark at 25°C with standard feed and Water *ad libitum*, per the recommendations established in (Agricultura and Ganadería, 2001).

In vitro evaluation: In all cases, a conditioning period of 4 days prior to start the experimental protocol was used. After abdominal dissection the ileum was obtained, tissues were maintained in Ringer-Krebs-Henseleit solution and were cleaned of adjacent tissue. The segments of 0.5cm were obtained, which were stabilized at one gram for 30min. The spasmolytic activity assay was performed as described by (Estrada-Soto *et al.*, 2010). Spasmolytic effects exhibited by the test samples (0.1→1000µg/mL) and controls (Papaverine, 0.3→300µg/mL) were compared respect to the basal state (5min) of the normal spasmodic activity, for this, Acqknowledge software (BIOPAC®) was used.

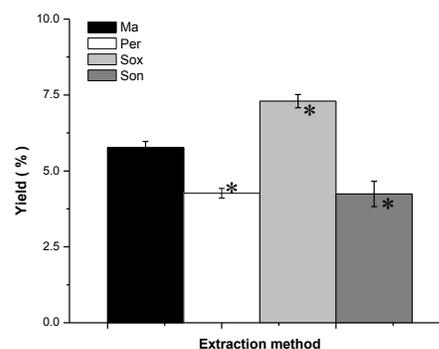
Statistical analysis: All results were stored in an Excel-Microsoft Office® database and processed using Origin® Microcal 8.0. All assays were carried out in triplicate and results are expressed as the mean±standard error. Normality of the data, analysis of variance (ANOVA) was performed followed by a Tukey test; values of $p<0.05$ were considered statistically significant. Pearson correlation coefficient (r) was employed to measure strength of a linear association between temperature of extraction and pharmacological effect induced by all extracts derived from *B. crassifolia*.

Results

Under these experimental conditions, *B. crassifolia* leaves extraction rate is higher via sonication (m: 0.032 ± 0.004 ; $r^2:0.96\pm0.02$) with respect to maceration (m: 0.022 ± 0.001 ; $r^2: 0.94\pm0.004$), soxhlet (m: 0.012 ± 0.003 ; $r^2: 0.84\pm0.05$) and percolation (m: 0.006 ± 0.001 ; $r^2: 0.92\pm0.02$). The cumulative extraction allows identifying that in all cases the extraction efficiency was higher than 99% (**Graph 1**) after three extractions; in this sense, a net yield of the soxhlet, maceration, percolation and sonication extraction was 7.29 ± 0.21 , 5.77 ± 0.2 , 4.27 ± 0.16 and $4.24 \pm 0.42\%$, respectively (**Graph 2**).



Graph 1. Dynamics of natural products extraction from *B. crassifolia* leaves according to the extraction method employed. The results are expressed as the mean±standard deviation of three experiments (* $p<0.05$ vs. soxhlet).

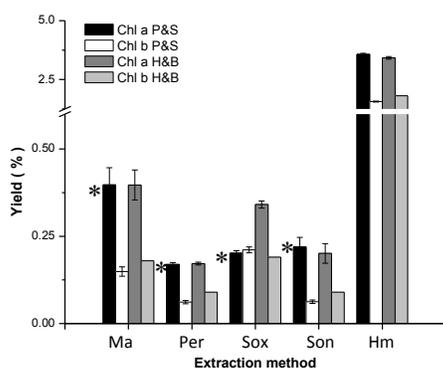


Graph 2. Net yield of natural products extraction of *B. crassifolia* leaves according to the method used: maceration (Ma), percolation (Per), soxhlet (Sox) and sonication (Son). Results are expressed as the mean ± standard deviation of three experiments (* $p<0.05$ vs. maceration).

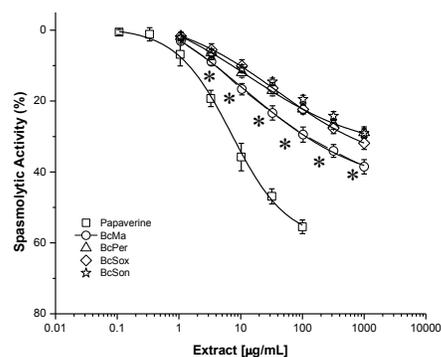
Pearson and Strickland (P&S) methodology allowed to determine a significantly ($p<0.05$) higher content of chlorophyll-a and -b by maceration (0.4 ± 0.05 and $0.15\pm0.01\%$) with

respect to soxhlet (0.2 ± 0.01 and 0.21 ± 0.01 %), percolation (0.17 ± 0.01 and 0.06 ± 0.005 %) and sonication (0.22 ± 0.03 and 0.06 ± 0.005 %) extraction. Likewise, the Hager & Meyer-Bertenrath (H&M-B) methodology allows estimate a significantly higher content of chlorophyll-a and -b by maceration (0.4 ± 0.04 and 0.18 ± 0.02 %) with respect to extraction via soxhlet (0.34 ± 0.01 and 0.19 ± 0.01 %), percolation (0.17 ± 0.001 and 0.09 ± 0.01 %) and sonication (0.2 ± 0.03 and 0.09 ± 0.01 %). Analysis of the acetone 90% extract of *B. crassifolia* revealed a $>3.5\%$ and $>1.5\%$ of chlorophyll-a and -b content (**Graph 3**).

Pharmacological evaluation in rat ileum strips allowed identify that *BrMa*, *BrPer*, *BrSox* and *BrSon* reduce the spontaneous contractions (E_{max} : 38.54 ± 2.03 , 28.9 ± 1.6 , 31.87 ± 1.79 , and 29.09 ± 1.27 %; EC_{50} : 11.6 ± 2.38 ; 13.22 ± 2.83 ; 39.74 ± 2.64 and >100 $\mu\text{g/mL}$, respectively) of ileum in a concentration-dependent manner. However, the effects were significantly lower than papaverine (E_{max} : 59.32 ± 2.57 %; EC_{50} : 7.11 ± 1.0 $\mu\text{g/mL}$; **Graph 4**).



Graph 3. Estimation of chlorophyll a (Chl a) and b (Chl b) present in *B. crassifolia* by P&S and (M&H-B) methods in extracts obtained by maceration (Ma), percolation (Per), soxhlet (Sox) and sonication (Son). Estimation of chlorophylls with acetone was included. The results are expressed as the mean \pm standard deviation of three experiments (* $p < 0.05$ vs. Hm)



Graph 4. Spasmolytic effect of *B. crassifolia* extracts (Maceration, *BrMa*; Percolation, *BrPer*; Soxhlet, *BrSox* and Sonication, *BrSon*; $0.1 \rightarrow 1000$ $\mu\text{g/mL}$) in ileum strips isolated from rats. Results are expressed as the mean \pm SEM of six experiments (* $p < 0.05$ vs. Papaverine).

Discussion

The methods of extraction used in Pharmacognosy and Natural Products are widely documented and their procedures may be in function of particle size, temperature, solvent, time of contact plant material / solvent, among other factors (Kaufmann and Christen, 2002). On the other hand, the secondary metabolites contained in the organic or aqueous

extracts show physicochemical differences which may be related to the extraction process (Chemat *et al.*, 2012). Under conditions employed, consecutive extraction of *B. crassifolia* leaves and adjustment to a nonlinear model indicates differences in extraction dynamics as well as in the net extraction yield.

Differences in extraction yields could be related to the energy of the extraction system. It has been reported that temperature plays a crucial role in the solubility, viscosity and density of natural product extracts (Cepeda and Villarán, 1999). It has been reported that the increase in temperature favors the kinetic energy of the molecules (Tan, 2014) present in the plant material as well as the solvent employed, this situation could increase the amount and the diversity of the secondary metabolites present in the extracts. Determination of chlorophylls present in a photosynthetic organism is an important measure in itself as an indicator of the physiological state of the plant species (Pinto *et al.*, 2001), in fact, different methods, procedures and mathematical models for the estimation of chlorophylls (APHA/AWWA/WEF, 2012) are reported. In order to have an approximation to potential variations in the content and amount of secondary metabolites present in the different preparations, the estimation of chlorophylls allowed recording the changes in the quantity and chlorophyll a/b ratio according to the method used. The lowest variation in the estimate of chlorophyll a and b is recorded by maceration (2.7/1 and 2.2/1), respectively. On the other hand, soxhlet extraction recorded changes in the estimation of chlorophylls (0.9/1 and 1.8/1), events that could be related to the temperature applied to the extraction system.

There are numerous experimental models and reports indicating that extracts derived from medicinal plants have a spasmodic activities (prokinetics, laxatives, spasmogenic and spasmolytic) on the gastrointestinal tract (Rahimi and Abdollahi, 2012). In fact, there are approximations about the mechanism of action by which natural products exert the spasmodic effect (Gilani *et al.*, 2005). In this sense, data indicated that crude extract of *B. crassifolia* obtained via maceration (*BcMa*) contains spasmolytic constituents and exerts a relaxing effect of spontaneous contractions in a concentration-dependent manner and can be considered as a partial agonist agent. In this sense, flavonoids and polyphenolic compounds (Da Rocha *et al.*, 2012), tannins (Sieniawska and Baj, 2017), proanthocyanidins (Kruger *et al.*, 2014) are reported with smooth muscle activity; however, it requires more studies in order to identify the secondary metabolite(s) involved in the relaxing effect as well as its mechanism of action.

Conclusion

Factors such as temperature, time, solvent, extraction method, among other factors, influence the amount and diversity of secondary metabolites contained in extracts of *B. crassifolia*. The estimation of chlorophylls allows recording the variability of the chlorophyll content and according to the method of extraction used, to be evidence of the modification of the metabolic content present in an extract. Finally, extract of *B. crassifolia* obtained by maceration (BcMa) exerts spasmolytic activity and is the ideal method to development pharmacological studies as well as bio-guided phytochemical studies.

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