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EVALUATION OF EMERGING METHODS ON THE POLYPHENOL CONTENT, ANTIOXIDANT CAPACITY AND QUALITATIVE PRESENCE OF ACETOGENINS IN SOURSOP PULP (*Annona muricata* L.)[1]

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ABSTRACT- The aim of this study was to obtain extracts from soursop pulp that were obtained with different solvents (chloroform, methanol, ethyl acetate and water) and different extraction methods (soxhlet, sonication and microwave), and analysed their extractable polyphenol content, antioxidant capacity and qualitative presence of acetogenins. The most efficient extraction method to obtain extractable polyphenols with high values of scavenging capacity by DPPH was sonication followed by microwave when methanol was used. The acetogenins were detected only in chloroform and ethyl acetate extracts obtained by the three extraction methods. Sonication or microwave was effective to obtain acetogenins or phenolic extracts in greater quantity than reported in soursop pulp, in a short time and few solvent.
Index terms: *Annona muricata*, emerging extraction methods, bioactive compounds.

AVALIAÇÃO DE MÉTODOS EMERGENTES SOBRE O CONTEÚDO DE POLIFENÓIS, CAPACIDADE ANTIOXIDANTE E PRESENÇA QUALITATIVA DE ACETIOGENINAS EM PULPA DE SOURSOP (*Annona muricata* L.)

RESUMO- O objetivo deste estudo foi avaliar os extratos do polpa da graviola obtidos com diferentes solventes (clorofórmio, metanol, acetato de etilo e água) e diferentes métodos de extração (Soxhlet, sonicação e micro-ondas). Foi analisado o conteúdo de polifenóis extraíveis, capacidade antioxidante e presença qualitativa de acetogeninas. O método de extração mais eficiente para obter polifenóis extraíveis e valores elevados de capacidade antioxidante (DPPH) foi por sonicação, seguido por micro-ondas, quando o metanol foi usado. As acetogeninas foram detectadas somente em extractos com clorofórmio e acetato de etilo para todos os métodos de extração avaliados. Sonicação ou micro-ondas foi eficaz para extrair acetogeninas ou polifenóis em maior quantidade do que o relatado em polpa de graviola, em pouco tempo e pouco solvente.
Termos para indexação: *Annona muricata*, métodos emergentes de extração, compostos bioactivos.

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The consume of soursop fruit has increased in the last years because in the pulp has been identified some bioactive compounds as polyphenols and acetogenins which are associated with prevention of diverse pathologies, such as neurodegeneration, cancer, diabetes, cardiovascular, and anti-inflammatory diseases (ANDRADE AND FASOLO, 2014; CORTÉS et al., 2014; GONZÁLEZ-ESQUINCA et al., 2014). On the other hand, Champy et al (2005) and Potts et al (2012) reported that isolated acetogenins of soursop pulp or other *Annonaceae* fruits caused neurodegeneration or atypical Parkinsonism. However these studied levels (i.e. levels of annonacin in blood or brain parenchyma) may not be extrapolated with their results, therefore the importance to study acetogenins in *Annonaceae* pulp is due to there are few studies on acetogenin content and it is required to consider if consumption of this fruits are a potential risk factor or not. Currently, the unconventional and emerging methods such as microwave and ultrasound sonication have been proposed as extraction methods of bioactive compounds. Cardoso-Ugarte et al. (2014) found that the extraction of betalains in red beet (*Beta vulgaris*) by microwave-assisted extraction were two times higher than those obtained during conventional extraction at 80 °C. Paz et al. (2015) demonstrated that ultrasound technology is very efficient for the extraction of polyphenols from native plants found in the mexican desert. However the use of emerging methods in extraction of acetogenins has not been reported. Therefore is important to investigate alternative extraction methods to quantify bioactive compounds using emerging methods to reduce extraction times and avoid oxidation reactions. The aim of this study was to evaluate different emerging methods and solvents to extract polyphenols and acetogenins from soursop pulp. Polyphenol antioxidant capacity was evaluated as well.

Soursop fruit (*Annona muricata* L.) was harvested at physiological maturity in orchards located Nayarit, Mexico. The fruit was stored (25 °C) until ripening (15-19 °Brix). The samples were frozen at -70 °C, followed by lyophilisation at -50 °C and a pressure of 0.12 mbar using a LABCONCO (Model 77522020, Kansas M., E.U.) freeze dryer. Polyphenols and acetogenins were extracted with four solvents: chloroform, methanol, ethyl acetate and water. Soxhlet extraction was performed on a Soxhlet Novatech (Model VH-6, Mexico), 10 g of sample were placed in the extraction trap and in the ball flask were placed 100 mL of each solvent. The extraction started at a boiling temperature for each solvent (60-85 °C) for 24 h (ASPÉ AND FERNANDEZ, 2011). Sonication extraction was carried out using 10 g of sample combined with 100 mL of each solvent at 25 °C. Subsequently, the sample was treated with 3 cycles in every hour for 3 h at a constant frequency of 42 kHz, on a Cole-Parmer ultrasonic equipment (model 8891, Illinois E.U.) (ASPÉ AND FERNANDEZ, 2011). Microwave extraction was conducted with 10 g of sample wich were placed in an Erlenmeyer flask containing 100 mL of each solvent. The flask was placed in the chamber of a microwave oven (SAMSUNG model MW830WA, Selagnor, Malaysia) operating at 600 W. The extraction was made with irradiation cycles of 10 s for 1 min (PADMAPRIYA et al., 2012). All extractions were performed in triplicate and extracts were filtered and concentrated on a rotary evaporator (Yamato, model RE300, Japan) at a temperature of 30-50 °C at 90 rpm. Extractable polyphenols (EPPs**)** content was quantified using the Folin-Ciocalteu reagent following the method of Montreau (1972). EPPs content was expressed as mg of gallic acid equivalents (GAE) 100 g^{-1} dry matter (DM). The method of ferric-reducing antioxidant power (FRAP) assay described by Benzie and Strain (1996), the 2-diphenyl-1-picryhydrazyl (DPPH) radical-scavenging assay described by Sánchez-Moreno et al. (2005) and ABTS (2,2-Azinobis-3-ethylbenzotiazoline-6-sulfonic acid) radical scavening essay (RE et al., 1999) were used. A standard curve of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was employed to estimate the antioxidant capacity as µM Trolox equivalent (µMTE g^{-1} DM). Each extract was subjected to a thin layer chromatography (TLC) to determine the presence of acetogenins (GU et al., 1995). Anonnacin standard (Biobhiopha-BBP02455) was used as a positive control.

The polyphenol content in the extracts is shown in Figure 1. Methanolic extracts showed the highest content of EPPs with values of 3.11-3.56 g 100 g^{-1} DM, followed by ethyl acetate (1.93-2.27 g 100 g^{-1} DM), water (1.56-1.80 g 100 g^{-1} DM) and chloroform (1.95-2.02 g 100 g^{-1} DM) extracts. Polyphenols have a large number of unsubstituted hydroxyl groups or sugars considered polar molecules, so they are soluble in polar solvents such as methanol, ethanol, acetone or water. On the other hand, there are a few proportions of polyphenols with low polarity (some types of flavonoids) that can be extracted with chloroform, dichloromethane, diethyl ether or ethyl acetate (GONZALEZ-MONTELONGO et al., 2010). The extraction method had a significant effect on EPPs (Figure 1). The extraction with sonication and

microwave resulted in high levels of EPPs (3.42 and 3.56 mg 100 g^{-1} DM, respectively), which was 24% more extracted EPPs quantity compared with Soxhlet extraction. The extraction of bioactive compounds using sonication is reached by the vibration of sound waves, which causes the acceleration of particles, which in turn induce to the solute to quickly pass to the solvent increasing its diffusivity (LIAZID et al., 2011). In the case of the extraction with microwaves, the energy is absorbed efficiently by compounds contained in the plant material, therefore, the inner temperature dramatically increases and facilitates mass transfer of secondary metabolites in the solvent, allowing a fast and efficient extraction (ZHANG et al., 2011). A similar effect to this work was found by Yingngam et al. (2014) who reported a more efficient extraction of polyphenols from *Cratoxylum formosum ssp. Formosum* leaves by ultrasound than conventional method by heating. The level of EPPs in the methanol extract of soursop pulp found in this study was almost two times higher than reported by other authors in soursop pulp, who have reported values from 1.86 g GAE 100 g^{-1} DM (MORENO-HERNÁNDEZ et al., 2014; SIQUEIRA et al., 2015). The values of antioxidant capacity determined by $DPPH^{\bullet+}$ and $ABTS^{\bullet+}$ radical scavenging assay are shown in Figures 2 and 3, respectively. High values of scavenging capacity for DPPH assay were observed in methanol polyphenol extracts (935.10-1033.18 µmol TE g^{-1} DM) obtained by the three extraction methods. These higher values coincided with their high contents of EPPs which could be attributed to the high polarity of the solvent that is more effective in the recovery of antioxidant compounds than non-polar solvents (DORTA et al., 2012). On the other hand, the values of antioxidant capacity found by DPPH essay using chloroform and ethyl acetate as extraction solvents, indicates that they could be more effective for extraction of lipophilic compounds such as nonpolar acetogenins, flavonoids and other lipophilic antioxidants reported in soursop pulp (CORREA et al., 2012; FLOEGEL et al., 2011). The determination of antioxidant capacity by ABTS assay demonstrated that methanolic extracts obtained by the three methods were the samples with the higher values (954.26-995.75 µmol TE g^{-1} DM), after aqueous extracts (905.03 µmol TE g^{-1} DM). This result is consistent with other reports where higher antioxidant capacity determined by ABTS method was obtained using methanol or water as an extracting agent (LABRINEA AND GEORGIOU, 2004). The highest value of antioxidant capacity determined either by $DPPH^{\bullet+}$ or $ABTS^{\bullet+}$ essay in polyphenol extracts was obtained by extraction with sonication using methanol as solvent, which is related to its high polyphenol content. Similar results were reported using ultrasound for extraction of polyphenols from grape pomace (GONZÁLEZ-CENTENO et al., 2015) where high values of antioxidant capacity were also related to the high polyphenol content. Antioxidant capacity with values of 2.88 µmol TE g^{-1} DM have been reported for soursop pulp by the DPPH essay and 4.8-12.78 µmol ET g^{-1} DM by ABTS assay (KUSKOSKI et al., 2005; LAKO et al., 2007), although it is difficult to compare antioxidant capacities because the differences in method and extraction conditions, this parameter was higher in this experiment. Polyphenols may be able to chelate metal ions such as iron and copper because of the large number of hydroxyl groups (OH^{+}) (SÁNCHEZ-VIOQUE et al., 2013). The effect of different extracts on ferrous ion-chelating is shown in Figure 4. The extracts obtained with methanol and ethyl acetate by sonication showed the highest values of chelating capacity (14.55 and 20.71 µmol TE g^{-1} DM). This result coincided with the previous antioxidant capacity reported by Correa et al. (2012) in polyphenol extracts from soursop pulp determined with the same test. The Table 1 shows the linear correlation between the measured antioxidant capacity and EPPs obtained for three extraction techniques. Correlation values between EPPs with the ABTS, DPPH a FRAP methods were between R^2=0.879 and R^2=0.999, when the extraction was done with methanol independently from extraction method. However, when the extraction was done with water or ethyl acetate, the correlation was not significant between EPPs and DPPH or EPPs and ABTS methods. It is could been due to that most simple polyphenols are soluble in polar solvents and a minor content of polyphenols is extracted with not polar solvents such as flavonoids (SULAIMAN et al., 2011). The correlations found between EPPs and antioxidant capacity (DPPH and ABTS assay) agrees with the reports of other authors, Floegel et al. (2011) found a strong positive relationship between antioxidant capacities determined by both scavenging assays (DPPH and ABTS) and total phenolics and flavonoids content in the 50 most popular fruits. The presence or absence of acetogenins by TLC is presented in Figure 5. The presence of acetogenins was observed in chloroform and ethyl acetate extracts with Rf values of 0.18 to 0.20, while in aqueous and methanolic extracts these compounds were not detected. The sonication method and microwave methods were most effective for extracting acetogenins according to the very intense pink color of bands compared with the

extracts from conventional method (Soxhlet) in the TLC. This result is very important because until now only the extraction of acetogenins by continuous heating at long periods of time (24 h) had been reported (MELOT et al., 2009). In soursop pulp has been reported an annonacin content of 0.023 mg/100 g pulp (POTTS et al., 2012). Also, it has been reported others acetogenins (SUN et al., 2014). The consumption limit of acetogenins in pulp has not been reported, therefore it cannot be said that consumption of soursop pulp is toxic or not, thus are required more researches. In this experiment only was identified the presence of total acetogenins.

With the use of emerging methods in combination con solvents it is possible to obtain a significant polyphenols and identified acetogenins from soursop pulp. Using sonication or microwaves with chloroform or ethyl acetate are obtained extracts with acetogenins, and using water and methanol is possible to obtain phenolic extracts free of acetogenins.

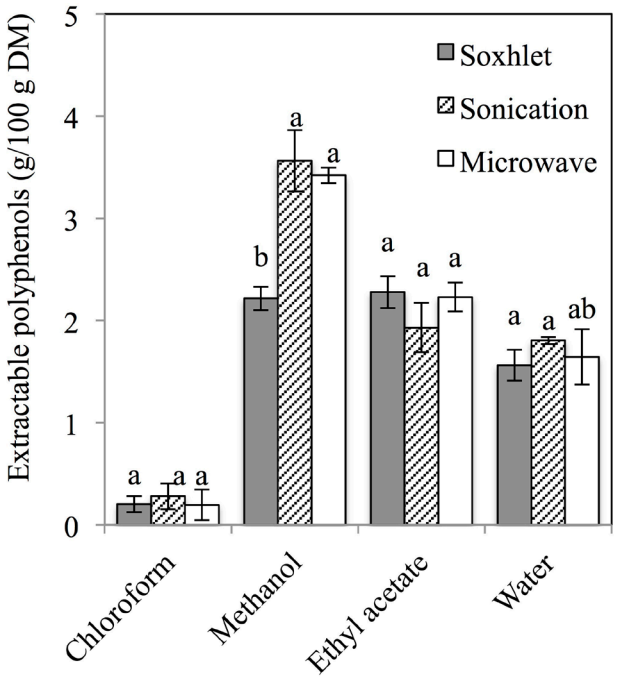

FIGURE 1-Extractable polyphenol content in soursop pulp extracts, obtained with four solvents and subjected to three methods of extraction.

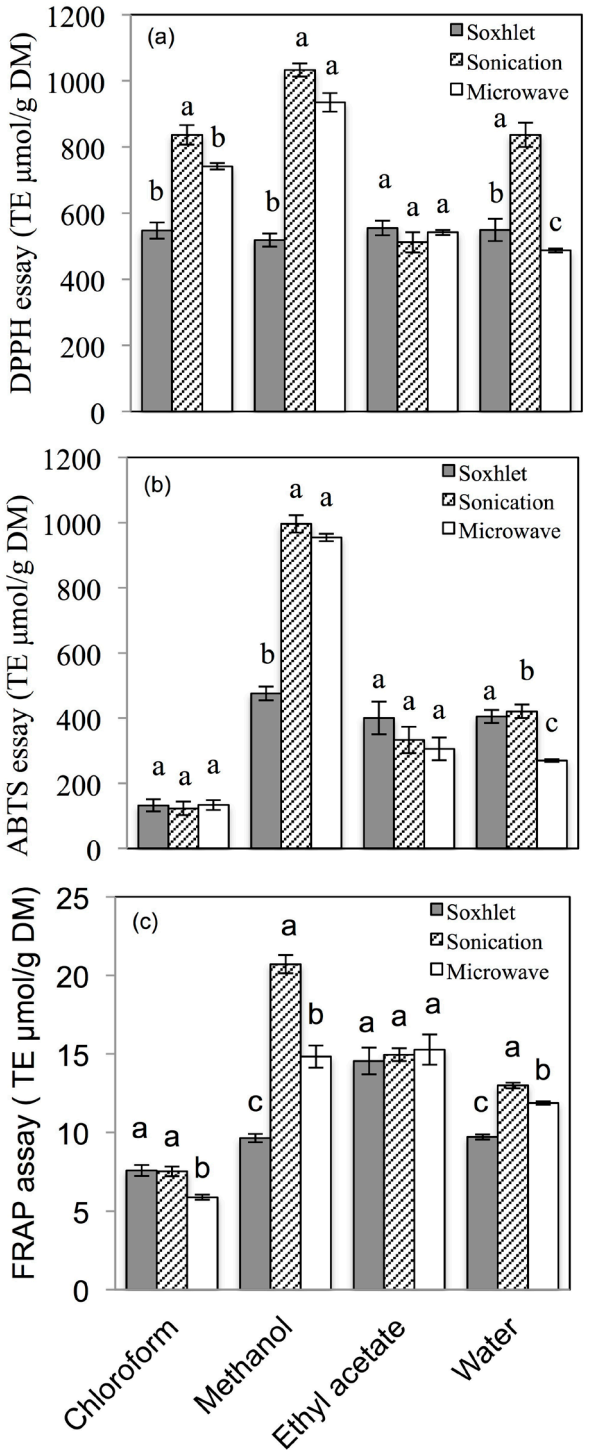

FIGURE 2- Antioxidant capacity by DPPH (a) and ABTS (b) radical scavenging essay and FRAP (c) method, in soursop pulp extracts, obtained with four solvents and subjected to three methods of extraction.

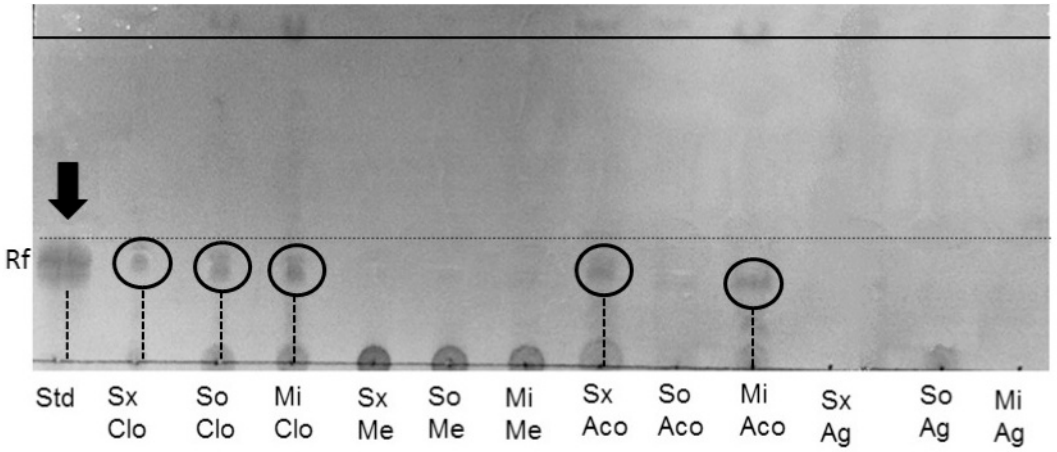

FIGURE 3 - Qualitative identification of acetogenins by TLC of extracts evaluated. Std = Annonacine standard, Sx = Soxhlet, So = Sonication, Mi = Microwave, Clo = Choloroform, Me = Methanol, Aco = Ethyl acetate and Ag = Water.

TABLE 1- Regression (R^2) and extinction coefficients between extractable polyphenols (EPPs), antioxidant capacity by DPPH, ABTS and FRAP assays in extracts of soursop pulp.

SOLVENT		**R^2**	**Extinction coefficient**
Chloroform			
	EPPs-DPPH	0.699	0.114
	EPPs-FRAP	0.817	0.087
	EPPs-ABTS	0.799	0.011
Methanol			
	EPPs-DPPH	0.990	0.011
	EPPs-FRAP	0.988	0.064
	EPPs-ABTS	0.879	0.004
Ethyl acetate			
	EPPs-DPPH	0.870	0.011
	EPPs-FRAP	0.602	0.232
	EPPs-ABTS	0.128	0.009
Water			
	EPPs-DPPH	0.175	0.061
	EPPs-FRAP	0.869	0.126
	EPPs-ABTS	0.346	0.158

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