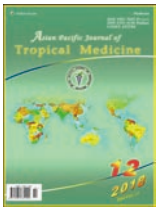

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Asian Pacific Journal of Tropical Medicine

journal homepage: www.apjtm.org

doi: 10.4103/1995-7645.248339

Ultrasound-assisted extraction of antioxidant polyphenolic compounds from *Nephelium lappaceum* L. (Mexican variety) husk

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ARTICLE INFO

Article history:
Received 28 May 2018
Received in revised form 30 August 2018
Accepted 20 October 2018
Available online 26 December 2018

Keywords:
Mexican variety rambutan
Ultra-sound assisted extraction
Antioxidant polyphenolic compounds
HPLC/ESI/MS

ABSTRACT

Objective: To reach the recovery and identification of antioxidant polyphenolic compounds from *Nephelium lappaceum* L. (Mexican variety) husk using ultrasound-assisted extraction and liquid chromatography/mass spectrometry as well as the *in vitro* antioxidant activity. **Methods:** Rambutan husk extracts were obtained by ultrasound-assisted extraction, mass/volume ratio, water/ethanol percentage and extraction time were evaluated. Once the best extraction condition of polyphenolic compounds was defined, a polyphenolic fraction was recovered using Ambetlite XAD-16. The total content of antioxidant polyphenolic compounds was determined by summation of the total hydrolysable polyphenol and total condensed polyphenol contents. Recovered compounds were identified by FTIR (ATR) spectroscopy and HPLC/ESI/MS. The antioxidant activity was carried out by ABTS, DPPH and lipid oxidation inhibition *in vitro* methods. **Results:** In Mexican variety rambutan husk, the total polyphenolic content was 487.67 mg/g, after ultrasound-assisted extraction. According to the HPLC/ESI/MS analysis 12 antioxidant polyphenolic compounds were identified, mostly ellagitannins such as geraniin, corilagin and ellagic acid. The antioxidant activity determined by ABTS, DPPH and lipid oxidation inhibition methods was demonstrated. The main functional groups of the identified compounds were determined by FTIR analysis. **Conclusions:** It was demonstrated that ultrasound-assisted extraction was effective and allowed the extraction and recovery of antioxidant polyphenolic compounds. Furthermore Mexican variety rambutan husk is an important source for recovering polyphenolic compounds with antioxidant activity, these compounds have potential application for the treatment/prevention of various diseases related to cancer and pathogenic microorganisms.

1. Introduction

Phenolic compounds constitute a wide group of chemical substances, are produced by plant secondary metabolism and are considered bioactive compounds[1]. Their biological properties give the bioactive activity, the interest for new medicine/supplements and high added value compounds[2]. Principal biological properties are antimicrobial activity[3,4], antidiabetic[5,6], anticancer[7], antiparasitic[8], among others. Polyphenols are present in different plants species, one of these with high phenolic content and few

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How to cite this article: Méndez-Flores A, Hérnandez-Almanza A, Sáenz-Galindo A, Morlett-Chávez J, Aguilar CN, Ascacio-Valdés J. Ultrasound-assisted extraction of antioxidant polyphenolic compounds from *Nephelium lappaceum* L. (Mexican variety) husk. Asian Pac J Trop Med 2018; 11(12): 676-681.

applications in the industry is the rambutan (*Nephelium lappaceum* L.). Rambutan is a red or yellow ovoid Malaysian tropical fruit, member from the Sapindaceae family[9], its husk represents 45.7% of its total weight[10] and is currently cultivated in some places of India, Indonesia, Thailand, Costa Rica, Guatemala and Mexico[11]. Rambutan varieties native from Malaysia and Indonesia were introduced to Mexico in the 1950s, the crop started in Chiapas and later was spread to other states (as Veracruz, Tabasco, among others)[11]. In Mexico, the pulp is the principal commercialized part of the fruit, it is consumed in fresh or it is used to obtain some processed products[12], however, this practice generates important quantities of not used wastes. Some reports declare that the rambutan husk contains phenolic compounds with different chemical structures and they are considered as bioactive. Other reports mentioned that rambutan husk polyphenols has antioxidant capacity, which are able to inhibit free radicals responsible of cellular oxidation[13]; inhibit the pathogenic bacteria *Streptococcus pyogenes*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* growth for mention some examples[14]; has antidiabetic activity by reducing the cholesterol and triglycerides blood concentrations and glycosylated serum protein[15]. Other studies demonstrated the cytotoxic effects of bioactive compounds against 4T1 breast cancer cells and mouse embryonic fibroblasts cells[16], inhibition of the human immunodeficiency virus (HIV) replication and antiparasitic activity against *Trypanosoma cruzi*, *Leishmania infantum*, among others.

Despite the multiple applications there are few reports about the extraction and identification of phenolic compounds present in Mexican variety rambutan husk, this subject is crucial to develop different extraction strategies that assure the bioactivity for these high added value compounds. The objective of this study was the recovery by ultrasound-assisted extraction of antioxidant polyphenolic compounds from Mexican variety rambutan husk, the identification of the recovered compounds by liquid chromatography/mass spectrometry and to determinate its antioxidant activity *in vitro*.

2. Materials and methods

2.1. Plan material

The Mexican variety rambutan husks (Soconusco region, Comitán, Chiapas, Mexico) were dehydrated at 50 ℃ for 48 h in a conventional oven[10], then was processed in a blade mill.

2.2 Extraction and purificaction of rambutan husk polyphenols

In order to define the best extraction condition of total polyphenolic compounds, experiments were performed under a completely randomized experimental design with factorial arrangement by ultrasound-assisted extraction; ANOVA and Duncan's test were done and all experiments were carried out by triplicate. Evaluated factors were mass/volume ratio (g/mL), ultrasound extraction time (min) and ethanol/water percentage (extractor agent), all experiments were performed at room temperature. Table 1 described the evaluated extraction conditions and the samples ID. With the best extraction condition identified, obtained extract was processed by liquid column chromatography with Amberlite XAD-16 to recover a polyphenolic fraction. Ethanol was evaporated and the polyphenols were recovered as a fine powder.

Table 1

Extraction conditions of polyphenolic compounds.

m/v ratio (g/mL)	Time (min)	% EtOH	Sample code
1:3	10	10	A1
		30	A2
		50	A3
	15	10	B1
		30	B2
		50	B3
	20	10	C1
		30	C2
		50	C3
1:5	10	10	D1
		30	D2
		50	D3
	15	10	E1
		30	E2
		50	E3
	20	10	F1
		30	F2
		50	F3
1:7	10	10	G1
		30	G2
		50	G3
	15	10	H1
		30	H2
		50	H3
	20	10	I1
		30	I2
		50	I3

m/v: mass/volume ratio.

2.3. Determination of total hydrolysable polyphenols

Total hydrolysable polyphenols were determined using Folin's Ciocaulteu reagent[10]. Experiment was performed by triplicate, total hydrolysable polyphenols content was expressed as gallic acid equivalents (GAE).

2.4. Determination of total condensed polyphenols

Total condensed polyphenols were quantified using ferric reagent and HCl-butanol[10]. The experiment was performed by triplicate, total condensed polyphenols concentration was expressed as catechin equivalents.

2.5. ABTS antioxidant assay

For the formation of $ABTS^{+}$ radical, 2.45 mM potassium persulfate was mixed with 7 mM ABTS solution (1:2 v/v). The mixture was carried out and rest in the dark at room temperature for 12-16 h. Subsequently, absorbance was measured at 734 nm, the solution was diluted with ethanol until (0.70±0.02) absorbance. For this study, 10 μL of sample (rambutan husk polyphenols) were mixed with 1 mL of ABTS adjusted solution and the absorbance was measured. Water was used as control and the results were reported by inhibition

percentage (all experiments were carried out by triplicate).

$$\% \text{ inhibition} = \left[1-\left(\frac{A_{\text{sample}}}{A_{\text{control}}}\right)\right]\times 100\%$$

A= absorbance (nm)

2.6. DPPH antioxidant assay

The 2,2-diphenyl-1-picrylhydracyl (DPPH) free radical scavenging was quantified as follows. Briefly, DPPH (Sigma Aldrich Mexico ®) was prepared at 60 μM concentration in absolute methanol. Subsequently, 193 μL of DPPH-methanol solution were mixed with 7 μL of sample (rambutan husk polyphenols), the solution was allowed to stand for 30 min at room temperature, then the absorbance of solutions was determined by micro-plate reader (TECAN®Sunrise) at 517 nm. DPPH-methanol was used as a control. And the results were reported as inhibition percentage using the above mentioned equation (all experiments were carried out by triplicate).

2.7. Lipid oxidation inhibition (LOI) assay

For the assay, linoleic acid was employed as the lipid source. Linoleic acid solution was prepared with 0.35 g of linoleic acid and 0.937 g of Tween 20 in 5 mL of 96% ethanol. A total of 50 μL of sample (rambutan husk polyphenols) was mixed with 100 μL linoleic acid solution and 1.5 mL of 0.02 M acetate buffer pH 4.0. The mixture was homogenized and then incubated for 1 min at 37 ℃. Immediately, 750 μL of 0.5 mM $FeCl_2$-EDTA were added to induce the oxidation and the samples were incubated for 1 and 24 h at 37 ℃. After each incubation time, 250 μL of solution was mixed with 1 mL 0.1 M NaOH in 10% ethanol. Subsequently, 2.5 mL of 10% ethanol was added and the absorbance was measured at 232 nm, 10% ethanol was used as blank. Antioxidant activity percentage was calculated using the equation:

$$\% \text{ LOI} = \left(\frac{\triangle D_{\text{control}} - \triangle D_{\text{sample}}}{\triangle D_{\text{control}}}\right)\times 100\%$$

where: $\triangle D_{control}$ is the difference between the absorbance of the control (distilled water) after 24 h and 1 h of incubation, and $\triangle D_{sample}$ is the difference between the absorbance of the sample after 24 and 1 h of incubation (all experiments were carried out by triplicate).

2.8. FTIR analysis

Polyphenol powder recovered after Ambertilte XAD-16 chromatography was placed on a metal plate to carry out the spectrophotometric analysis in a Perkin Elmer Spectrum FTIR model 65, using 16 sweeps as operation condition.

2.9. Identification of polyphenolic compounds by HPLC/ESI/MS analysis

The analysis by high performance liquid chromatography (HPLC) was performed on a Varian HPLC system including an autosampler (VarianProStar 410, USA), a ternary pump (VarianProStar 230I, USA) and a PDA detector (VarianProStar 330, USA). A liquid chromatograph ion trap mass spectrometer (Varian 500-MS IT Mass Spectrometer, USA) equipped with an electrospray ion source was used. Samples (5 μL) were injected into a Denali C18 column (150 mm × 2.1 mm, 3 μm, Grace, USA). The oven temperature was maintained at 30 ℃. The eluents were formic acid (0.2 %, v/v; solvent A) and acetonitrile (solvent B). Applied gradient was: initial, 3% B; 0–5 min, 9% B linear; 5–15 min, 16% B linear; 15–45 min, 50% B linear. The column was washed and reconditioned. The flow rate was maintained at 0.2 mL/min and elution was monitored at 245, 280, 320 and 550 nm. The whole effluent (0.2 mL/min) was injected into the source of the mass spectrometer, without splitting. All MS experiments were performed in the negative mode $[M-H]^{-1}$. Nitrogen was used as nebulizing gas and helium as damping gas. The ion source parameters were: spray voltage 5.0 kV and, capillary voltage and temperature were 90.0 V and 350 ℃, respectively. Data were collected and processed using MS Workstation software (V 6.9). Samples were firstly analyzed in full scan mode acquired in the *m/z* range 50–2 000. MS/MS analyses were performed on a series of selected precursor ions.

3. Results

3.1. Determination of total polyphenolic content

Obtained extracts (Table 1) was used to analyze the total polyphenolic content. Total polyphenolic content was calcuted using the content of total hydrolyzable and total condensed polyphenols. Results were shown in Figure 1.

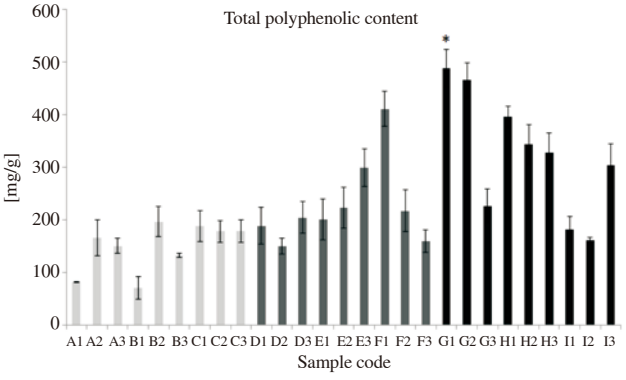

Figure 1. Total polyphenolic content obtained by ultrasound extraction of rambutan (mexican variety) husk. Vertical lines of each bar means standard deviation.

Treatment coded as G1(*), which corresponds to mass/volume ratio 1/7, extraction time 10 min and 10% ethanol/water was the best extraction condition of total polyphenols after ANOVA and Duncan's test (showed significant statistical differences at $P<0.05$). Using these conditions, the total polyphenolic content was (487.67±36.01) mg/g (Figure 1) of dry matter. Therefore, this extraction condition was selected to the subsequent experiments.

3.2. ABTS, DPPH and lipid oxidation inhibition assays

The results showed 92.501%, 73.730% and 91.740% of antioxidant activity by ABTS, DPPH, and lipid oxidation inhibition methods of rambutan husk polyphenols obtained with the best extraction condition (G1 treatment) and after Ambertlite XAD-16 chromatography was carried out (Table 2).

3.3. FTIR analysis

A FTIR analysis of the polyphenols obtained with the best extraction condition and after the Amberlite XAD-16 chromatography was carried out to identify the main functional groups of polyphenolic compounds. The signals of the main functional groups of polyphenolic compounds such as hydroxyl groups (3 235.69 cm^{-1}), carboxylic acids (1 702.49 cm^{-1}), aromatic rings (1 445.47 cm^{-1}) and carbonyl group (1 080.80 cm^{-1}) were determined as the most important. The signals are shown in Figure 2.

3.4. Identification of polyphenolic compounds by HPLC/ESI/MS analysis

Identification profile of polyphenolic compounds of Mexican variety husk was obtained by HPLC/ESI/MS. Compounds identification was carried out using negative ionization as MS operating conditions. Obtained results analysis from Mexican variety husk allowed 12 compounds identification. Figure 3 showed the chromatogram corresponding to the compounds separated by HPLC, compounds such as corilagin, geraniin and ellagic acid (compounds reported in Mexican variety husk) were identified. Table 3 showed the compounds identified by ESI/MS.

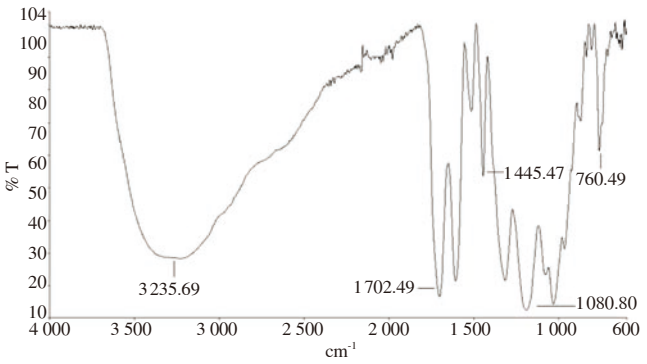

Figure 2. Signals of the main functional groups of polyphenolic compounds from rambutan (mexican variety) husk.

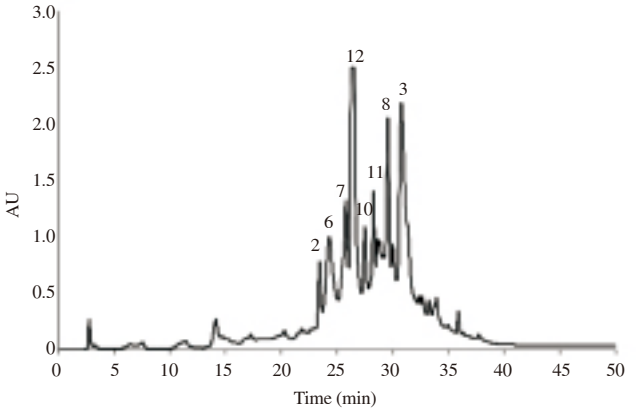

Figure 3. Chromatographic profile of main compounds in rambutan (mexican variety) husk.

Table 2

Antioxidant activities

Assay	Absorbance control	Absorbance sample	Inhibition (%)
ABTS	0.680±0.036 (at 734 nm)	0.051±0.011 (at 734 nm)	92.501±5.140
DPPH	0.310±0.008 (at 517 nm)	0.082±0.001 (at 517 nm)	73.730±2.950
Lipid oxidation inhibition	1.070±0.094 (at 232 nm)	0.088±0.021 (at 232 nm)	91.740±3.080

Abs= absorbance (nm).

Table 3

Identification of polyphenolic compounds in Mexican variety husk by HPLC/ESI/MS.

ID	R.T. (min)	Compound	$[M-H]^-$ (*m/z*)	MS2 (*m/z*)	Group
1	6.97	Gallic acid	169	125	Hydroxybenzoic acid
2	23.36	Brevifolin carboxylic acid	291	247, 248, 203	Ellagitannin
3	31.71	Ellagic acid	301	257, 229, 185	Hydroxybenzoic acid
4	2.72	Gallic acid 3-*O*-gallate	331	271, 169	Hydroxybenzoic acid
5	20.11	Isorhamnetin 3-*O*-glucoside 7-*O*-rhamnoside	623	477, 461, 315	Methoxyflavonols
6	24.23	Galloyl-HHDP-hexoside	633	463, 301, 249	Ellagitannin
7	25.71	Corilagin	633	481, 301, 275	Ellagitannin
8	29.53	Pedunculagin	783	301, 481, 765	Ellagitannin
9	14.02	Ellagic acid derivate	799	781, 479, 331, 301, 299	Hydroxybenzoic acid
10	27.44	Theaflavin 3,3'-*O*-digallate	867	715, 137	Theaflavins
11	28.25	Galloyl-bis-HHDP-hexoside (casuarinin)	935	657, 571, 463, 301	Ellagitannin
12	26.25	Geraniin	951	933, 301, 169	Ellagitannin

R.T.= retention time.

4. Discussion

To determinate the total polyphenolic content, we analyzed the contents of hydrolyzable and condensed polyphenols. According to our results, the maximum concentration of phenolic compounds was obtained using a mass/volume ratio 1/7, ultrasound extraction time 10 min and percentage ethanol/water 10% (sample coded as G1). This treatment showed significant differences (P<0.05) after ANOVA and Duncan's test were done and all experiments were carried out by triplicate. Reports indicated the factor that has a direct influence on polyphenolic compounds extraction is mass/volume ratio[17], because the compounds contained in the evaluated material show greater solute/solvent interaction and extraction rates may increase[18]. Respect to the compounds of Mexican variety husk, it has been reported that using mass/volume ratio of 1/5 around 500 mg/g (dry matter) of total polyphenols are obtained[10].

In the present study, the maximum concentration obtained was 487.67 mg/g. Although the values obtained in this study are lower than previously reported, they are higher compared to other reports with rambutan extracts of Asian origin. Some studies on the ultrasound-assisted extraction of compounds from rambutan not cultivated in Mexico reported total polyphenol content between 200 mg/g and 400 mg/g[19], these quantities are smaller than these quantities reported with Mexican variety husk. However, it has also reported higher amounts of polyphenols in some Asian varieties up to 700 mg/g[20].

The differences found in the polyphenolic contents between Mexican variety and the Asian varieties may be due to cultivation conditions, growth and development of the fruit, for example, climatic and soil conditions[21]. Temperature is a factor that also influences polyphenolic compounds extraction processes[22], generating a greater molecular interaction between the compounds and the extraction solvent. In this study the extraction of the compounds was at room temperature, in spite of this, results obtained here, were similar for the temperature extraction of Mexican variety rambutan compounds previously reported[10]. As mentioned, temperature is important for polyphenols extraction. For this study, the controlled temperature was not used, but ultrasound-assisted extraction technology was used. It has been reported that it is a technology that allows the compounds extraction in adequate yields[19], due to the effects of acoustic cavitation on the sample that cause a better solvent penetration and therefore a greater compounds release from the sample to the solvent[23].

The antioxidant activity of the recovered polyphenolic compounds was evaluated in this study. For the ABTS assay a 92.5% inhibition of this radical was obtained, this means that polyphenolic compounds recovered from Mexican variety rambutan husk showed a high antioxidant capacity. Other studies have reported antioxidant capacities using this radical, however the inhibition values have been lower, these differences are attributed to the evaluated samples origin, especially geographic origin, environmental crop conditions, polpyphenolic compounds concentration and extraction method[20]. Antioxidant activity was evaluated using another radical, DPPH. For this assay a 70.69% inhibition was obtained, this represents a good antioxidant activity using this assay and complements the results obtained with ABTS assay. It has been reported that to determine an adequate antioxidant activity analysis of polyphenolic compounds it is necessary to try both assays[24]. On the other hand, the inhibition of lipid oxidation was evaluated as a complementary analysis for antioxidant activity and an inhibition value of 91.74% was obtained. This test is important for this study because it allows to evaluate the ability of polyphenolic compounds to avoid the oxidation of biomolecules such as lipids, this means evaluated compounds have the potential to prevent human body diseases caused by cellular and molecular oxidation processes[13]. Most of these diseases are degenerative and cause more serious conditions such as the development of different cancer types. In this sense, it has been reported that polyphenolic compounds from rambutan peel are able to inhibit free radicals that related with the development of human mouth-epidermal carcinoma cells and colorectal adenocarcinoma cells (Caco-2)[25].

Once the antioxidant activity of polyphenolic compounds from Mexican variety rambutan husk was determined, compounds identification was carried out using HPLC/ESI/MS. This identification was complemented with FTIR analysis, where the signals of the main functional groups of polyphenolic compounds such as hydroxyl groups (3 235.69 cm^{-1}), carboxylic acids (1 702.49 cm^{-1}), aromatic rings (1 445.47 cm^{-1}) and carbonyl group (1080.8 cm^{-1}) were determined as the most important. Six ellagitannins were identified, of which, 4 have already been reported for Mexican variety rambutan husk[10] and two more were identified in this study. The ellagitannins are recognized for their antioxidant, antimicrobial, anti-cancer properties *etc.*[14,26,27], for that reason they are considered compounds with an important bioactive capacity and are considered as nutraceutical agents. For example, compounds corilagin, geraniin and ellagic acid have been evaluated against pathogenic microorganisms transmitted by food, specifically, against *Streptococcus mutans*, methicillin-resistant *Staphylococcus aureus*, *Escherichia coli* and fungus such as *Candida albicans*, pathogens that cause diseases in humans[28]. As mentioned above, these compounds also have anticancer capacity against cancer cell lines such as HeLa (cervical cancer)[29], against CLS-354 cells (mouth carcinoma cells)[30]. Their presence in Mexican variety rambutan husk have relevant importance because this material can be used to recover bioactive compounds with medical and pharmaceutical applications. Compounds 1, 4, 5, 6, 8 and 10 are described for first time in Mexican variety rambutan husk. These compounds also have an important bioactive potential; for example, compounds 1 and 4 have been reported as effective agents against the repression of hepatitis C virus, this means that they also possess antiviral activity and represent a potential application for treatment of viral diseases[31]. Compounds 5 and 10 have also been reported as important agents with bioactive potential as antioxidants and antimicrobials[32].

In conclusion, it was demonstrated that the ultrasound-assisted extraction was effective and allowed the recovery of the antioxidant polyphenolic compounds in similar levels to previous reports, it is confirmed that the Mexican rambutan variety husk represents an important source for the recovery of antioxidant polyphenolic compounds. In addition the identification of 12 antioxidant polyphenolic compounds with potential application in the medicine field (as treatment or prevention agents) was achieved.

Conflict of interest statement

We declare that we have no conflict of interest.

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