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Amaranth (*Amaranthus Hypochondriacus* L.): Use of Lignocellulosic Biomass to Obtain Antioxidant extracts Through Acid Hydrolysis

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Abstract: Amaranth is cultivated in Mexico and other countries because of its nutritional protein value. After its grain is harvested, roughly 550,000 tons per year of lignocellulosic biomass (LB) is generated. Amaranth LB (ALB) can be used as forage but its use is limited; mainly it is burned and consequently pollutes the air. Therefore, the objective of this work was to utilize the ALB, for extracting polyphenols, and processing ALB in order to obtain added value extracts with antioxidant activity (AOxA). ALB was oven dried and its fiber content was 21.0±1.3 g/100 g dry weight (dw). Total polyphenols content of an aqueous extraction obtained at 85°C for 5 min of dried ALB polyphenols was 7.6±0.56 mg gallic acid equivalent (GAE)/g dw. These extracts were called extractable polyphenols (EPP). The best conditions to obtain the called non EPP (NEPP) of ALB were obtained using the surface response method. The treatment using 5% (w/v) H_2SO_4 at 111°C for 90 min yielded 41, 2 mg GAE/g dw of TP. AOxA of EPP and NEPP expressed as their IC_{50} values assessed by the reduction of DPPH• method were 1.56 and 1.66 mg GAE/g dw, respectively. These extracts were also concentration dependent of Fe^{2+} at 0-1.4(GAE)/g dw and 0-0.009(GAE)/g dw, respectively. ALB may be processed to afford EPP and NEPP which may be an important natural commodity with AOxA for food industry.

Key words: Amaranth, lignocellulosic biomass, polyphenols, antioxidant activity

INTRODUCTION

Amaranth is an ancestral crop, its use was registered among the Mayas, the Aztecs and the Incas. In the zone of Tehuacán (Puebla), evidence of its use has been found since 4000 B.C[1]. The amaranth plant has wide leaves and, depending on the species, it has diverse pigments such as the amaranthine[2] It belongs to the caryophyllales order of the dicotyledons of the family Amaranthaceae. It includes more than 60 genus and 800 species of the annual herbaceous plants or perennial[3].

From the nutrimental perspective, amaranth has unique characteristics. Both the grain and the amaranth leaves have an exceptional protean quality, similar to the one of the milk's casein and other nutrients such as carbohydrates, lipids, minerals and natural vitamins such as B, C, B1, B2, B3; it also has folic acid, niacin, calcium, phosphorus iron, all needed for a balanced diet[1,4,5]. That is the reason why amaranth has become important in different countries such as the United States[6], Canada[7] and India[8], among others.

There are three species of the *Amaranthus* genus that produce great inflorescence: *A. hypochondriacus* and *A. cruentus*, that are cultivated mainly between Mexico and Guatemala respectively, and *A. caudatus*, that is specially cultivated in Peru[3,9,10]. In Mexico it is currently estimated that 3,729 hectares are cultivated nationwide with a production of 1.24 ton/ha[11]. The species *Amaranthus hypochondriacus*, also named *Amaranthus leucocarpus* S. Wats, is cultivated in Santiago Tulyehualco. After threshing, approximately 550,000 tons per year of amaranth lignocellulosic biomass are generated (ALB). Only a small portion is used as fodder for cattle, due to the presence of some metabolites in the ALB that are known as antinutritional factors[12,13] such as phytates and saponins, among others. The ALB is mostly burned outdoors, generating a serious air pollution problem.

On the other hand, it is known that ALB's cell wall is constituted by three structural components: cellulose, hemicellulose and lignin. Small amounts of mineral salts and diverse low molecular weight compounds are also present, and they have the characteristic of being water soluble or organic soluble (waxes, fats, tannins, phenols, terpenes, proteins, monosaccharides, oligosaccharides, pectic substances[14] (Figure 1).

There has been a lot of research focused on the study of plant extracts such as *Artemisia campestris* L, *A. herba halba*, *A. arboresens* L, *A. arvensis* L, *Juniperus oxycedrus* L, *Globularria alypum* L, *Oudneya africana*, *Thymeelaea hirsuta*, *Ruta monata* L, *Thapsia garganica*, *Teucrium polium* L, *Lauri folium*, *Althaeae radix*, quinoa and buckwheat[15-, 17], from where Extractable Polyphenols (EPP) [18] are obtained, showing important antioxidant properties, evaluated in different Redox systems. Nevertheless, when EP are isolated, there are residues that are disposed of.

In these residues, there is an important amount of lignin that is made of different acidic phenolic compounds such as: cinamic, p-coumricod, 3, 4-dioxycinnamic, ferulic, sinapinic, among others[2]. In order to access these polyphenols, it is necessary to hydrolyze this residue using acids or strong bases in order to break the links throughout the cell wall's polymeric net. The resulting hydrolysates are known as Non Extractable Polyphenols (NEPP), named that way for the first time by Arranz and col.[18].

Currently, there is a focus in research on the role of the polyphenolic compounds as antioxidants, antimutagenetic and free radicals scavengers that contribute defenses against the oxidative stress and are considered helpers and/or preventers of degenerative-chronic diseases such as cancer, cardiovascular illnesses and diabetes[19.20.] For this reason there is an increase in the demand for including these components

in the human diet, and at the same time it has forced the food industry to look for new sources of obtaining natural antioxidant compounds.

This research is focused on the conditions of aqueous extraction of EPP and hydrolysis to obtain the NEPP of CW-BCA from amaranth ALB. The total content of polyphenols and their AOxA are also discussed.

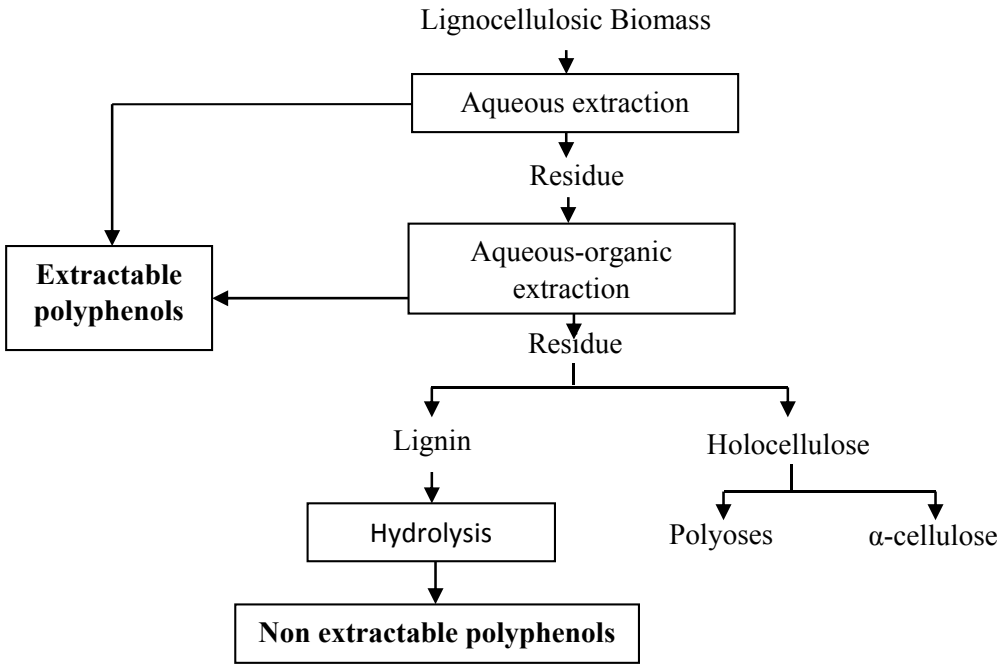


Fig. 1: Diagram of the Extractable Polyphenols (EPP) and Non Extractable Polyphenols (NEPP) extraction from the lignocellulosic biomass.

METHODS

Materials: The ALB was collected from *Amaranthus hypochondriacus* L. amaranth producers in Tulyehualco, México City in November 2012. The reagent Folin-Ciocalteau, the radical 2,2-diphenil-1-picrilhidrazil (DPPH), the cationic radical ABTS persulfate, iron chloride, Sigma-Aldrich ferrozine were used there. Methanol analytical standard.

Chemical characterization of amaranth ALB: Pretreatment. ALB was cleaned and milled in order to obtain flour that passed through a sieve number 40 and was retained on sieve 60, making sure that the particle size was between 250 to 420µm or less. The chemical analysis was performed in two stages. In the first one, the official methodology of the A. O. A. C. (2000) was followed in order to determine the humidity, ashes, crude protein, crude fiber, ethereal extract and N-free extract (by difference). The second stage was performed following the TAPPI (Technical Association of the Pulp and Paper Industry) official methodology (applicable also to lignocellulosic non-timber materials). Preparation of the sample (T 264 cm 1997), ashes (T 211 om 1993), extracted with organic solvents and hot water (T 204 cm 1997, T 207

cm 1999), Klason lignin (T 222 om 1998), holocellulose (Wise-1946), pentosans (UM-236-1939, T223-cm1984) α-cellulose (T 9 m-541954). The determinations were performed by triplicate.

*Water extraction:*The EP extraction is based on the method proposed by Miliauskas[21]. The extraction was carried out with distilled water 1:20 (p/v). Two extraction conditions were tested: cold: t=4h, T=25°C; hot: t=5min, T= 87°C. The extracts were filtered and centrifuged at 3000rpm during 15 minutes. The supernatant was separated and kept in jars at 4ºC for further analysis.

*Optimization of the acid hydrolysis:*To obtain the NEPP, an acid hydrolysis was carried out in two stages, according to the proposed method by Conde[22]. In the first one, the ALB sample was mixed with sulfuric acid 2.5, 5 y 7.5% (v/v) at 1:16 (p/v). The hydrolysis reaction was done in an autoclave at 80ºC during 30, 60, 90, 120 and 150 min. The resulting hydrolysate was neutralized (pH 7) with a saturated solution of sodium carbonate. After centrifugation at 5000 rpm during 10 min, NEPP was extracted with ethyl acetate (1:20 v/v). In the second stage, the previous steps were followed, only modifying the hydrolysis conditions and the sulfuric acid concentration at 5% and T= 90, 100, 110, 120 y 130°C. The solvent was recovered in a rotavapor Yamato RE200.

*Determination of total phenolics:*Total phenolics content (TP) was determined colorimetrically using Folin-Ciocalteu reagent, as described by Singleton[23], with modifications. Total phenolic assay was conducted by mixing 8.8 ml of de-ionised water, 0.5 ml of extracts, 0.5 ml saturated solution of Na_2CO_3 and 0.2 ml Folin–Ciocalteu reagent. The mixture absorbance was measured at 725 nm. A standard curve was prepared with gallic acid. Final results were given as mg gallic acid equivalent (GAE)/g dw.

*Determination of DPPH radical free scavenging potential activity:*DPPH radical free scavenging potential activity was measured according to the method of Brand-Williams[24]. 300 µL of DPPH solution was mixed in methanol (0.5 mM) with 500µL of the sample. 3.0 mL of methanol were added. The discoloration kinetic was monitored at 517 nm during 45 minutes; the percentage of the radical's inhibition was calculated with the following equation 1.

$$\%\ inhibition = \left[\frac{A_0 - A_1}{A_0}\right] \times 100 \quad (1)$$

A_0 and A_1 correspond to absorbances at 517 nm of the radical at the beginning and at 45 min, respectively. Concentrations of the phenolic compounds in the reaction medium were plotted against the percentages of the remnant DPPH• at the end of the reaction, in order to obtain the EC_{50} index, defined as the amount of antioxidant needed to decrease the initial DPPH• concentration by 50%.

Determination of iron ion chelating capacity:Chelation of iron ion (Fe^{+2}) was measured according to Xie [25] and compared with that of EDTA: 1 mL of the sample was mixed with 50 µL of iron (II) chloride 2mM and 1.85 mL of deionized water. After the reaction settled for 3 minutes, 100 µL of ferrozine 5 mM was added mixing vigorously. Absorbence at 562 nm was measured after 10 minutes at room temperature, and the chelating percentage was determined by the equation 2.

$$\%\ iron - chelating = \left[\frac{A_0 - A_1}{A_0}\right] \times 100 \quad (2)$$

Where A_0 is the absorbance of the control and A_1 is the absorbance in the presence of the extract sample.

Statical analysis: All experiments were conducted by triplicate and statistical analysis ANOVA was completed using the Statistical Package for Social Science (SPSS) program. Optimization was done with Statgraphics. Results were expressed as a mean of three determinations ± sd.

RESULTS

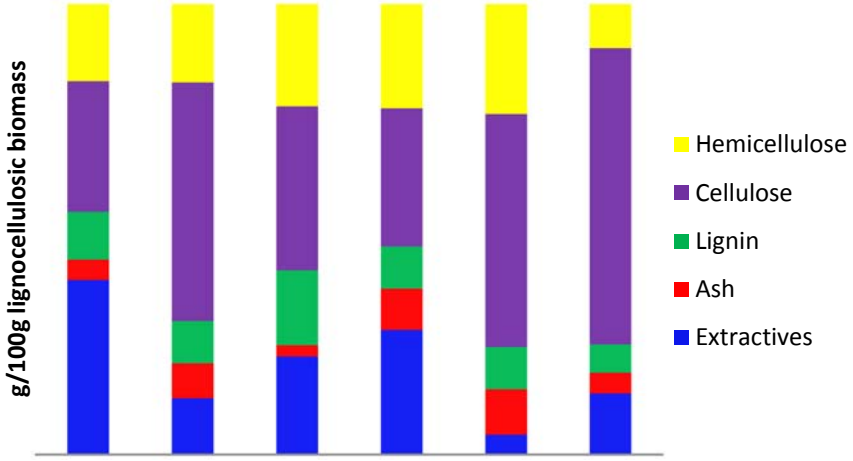
Chemical analysis of ALB :In table 1, the contents of dry matter, moisture, crude protein, crude fat, crude fiber and carbohydrate are shown, from samples of: banana stem (BS), residues cotton industry (RCI) obtained by Yawadio *et al.*[26] corn husk (CH), bean Straw (BSt) y sugarcane bagasse (SB) obtained by Coradi *et al.*[27] ALB. Here, it is shown that Bst contains 3.3 times more protein than ALB, while all the samples contain small amounts of fat.

Table 1: Chemical Analysis (A.O.A.C) of agro-industrial residues

Lignocellulosic biomas	Dry matter (%)	Moisture (%)	Crude protein (%)	Crude fat (%)	Crude fiber (%)	Carbohydrate* (%)	Reference
ALB	95.5±1.64	4.5±0.38	8.4±0.33	2.5±0.28	21.0±1.3	57.2	Experimental results
BS	91.6	8.4	8.6	2.3	44.6	28.3	[26]
CH	89.01	10.99	1.41	0.11	32.89	52.11	[27]
BSt	89.41	10.59	27.91	1.15	37.37	13.79	[27]
SB	92.15	7.85	6.95	3.52	20.02	39.13	[27]
RCI	91.5	8.5	6.9	1.8	56.7	21.5	[26]

****calculated by difference.*** **ALB= Lignocellulosic biomass amaranth crop, BS= Banana stem, CH= Corn husk, BSt= Bean straw, SB= Sugarcane bagasse, RCI= Residues cotton industry.**

All the residues are high in fiber and carbohydrates, which limit their use as a source of all the other compounds for the alimentary industry. In Figure 2, the contents of ash, lignin, α-cellulose and hemicellulose of ALB. Chemical composition data of BS y RCI were reported from Yawadio *et al*[26]; CH and BSt from Coradi *et al.* [27] and Olofsson *et al.* [28]; SB chemical composition was taken from Ferrer *et al.* [30] and Pernalete *et al.*[28]. From here, it can be observed that ALB ash content lays in the middle of the reported ranges. With regards of the lignin content, both ALB and corn husk show the highest values, whilst sugarcane bagasse and cotton industry residues are the ones that present a higher content of cellulose. The ALB hemicellulose content is comparable to BS and slightly lower than other important lignocellulosic residues, such as CH, BSt and SB. From the total ALB hemicellulose fraction, a calculation reveals that about 60% may be pentosans (xylose and arabinose), which suggests that ALB can be considered a potential raw material to obtain xylose, which is known to have a prebiotic effect on bifidobacteria in the digestive tract[31].



Cellulose= Calculated from hemicellulose, BS= Banana stem [26], CH= Corn husk[27, 28,] BSt= Bean Straw[27] [27], SB= Sugarcane bagasse[29,30], RCI= Residues cotton industry [26], ALB= Lignocellulosic biomass amaranth crop (Experimental results)

Fig. 2: Chemical Analysis (TAPPI standard methods) of agro-industrial residues.

The total ALB extractives content (ethanol-benzene 13.5±0.4 g/g dw, ethanol 9.8±0.45 g/g dw and water 20.7±0.8 g/g dw) is higher ($P<0.005$), which indicates that the ALB could be the best material for the extraction of EPP in comparison to the other lignocellulosic residues (LR).

Aqueous extraction conditions effects on ALB: In table 2, EPP content is shown, which was obtained following two set of extraction conditions. It is observed that the extraction at 87°C yielded only a 10% higher level of EPP than at lower temperature (25°C), showing that there was no significant difference from the extraction method employed.

The EPP content obtained from ALB is higher in comparison to the ones obtained from two types of LR used in Venezuela; LR 1=*Albizia caribaea* (2.2 (GAE)/g dw), LR 2=*Cassia grandis* (5.1 (GAE)/g dw), values reported by García and Medina[33]. Also, EPP from ALB is twice the values reported for *Amaranthus cruentus* v., *Rawa*, *Chenopodium*, quinoa and *A. cruentus v. Aztek* (3 (GAE)/g dw, 3.75 (GAE)/g dw and 2.95 (GAE)/g dw respectively) according to Pawel Pasko[34].

Table 2: Extractable polyphenols (EPP) present in ALB aqueous extracts

	Temperature (ºC)	Total phenolics ((GAE)/g dw)
ALB	25	6.9±0.31[a]
	87	7.6±0.56[a]

Values not sharing common letters are significantly different ($P<0.005$)

From the above results, it can be concluded that any of the employed methods for the extraction of EPP from ALB is sufficient to obtain a significant amount of PT, in comparison with other similar forage materials. When the ALB is used as forage for cattle, the release of EPP in the digestive tract of the ruminants is assured, however due to its anti-nutritional compound content, its use for this end is limited. If the EPP is required for human consumption, its aqueous extraction at 87°C is recommended, since at this temperature the anti-nutritional compounds will be eliminated. The nutritional importance of EPP is that these compounds are capable of dissolving in the gastrointestinal tract and therefore their bioavailability will allow them to cross the mucous intestinal barrier and be metabolized to bring their benefits to different levels [35]. The use of EPP in the alimentary industry brings about certain advantages such as polarity and stabilization of the products against oxidation, thus enhancing shelf life.

3.3 Optimization of the acid hydrolysis on ALB : The EPP aqueous extraction referred to above is also a preparative method to perform the acid hydrolysis. The acid hydrolysis breaks down the cellulose-hemicellulose-lignin bonds causing the release of base units of these polymers, altering the residual lignin composition, as well as enhancing the porosity of the ALB.

The entrance of water molecules into the inner biomass layers is eased [36], helping the interaction with the NEPP moieties that constitute lignin. In the first stage of the optimization of the hydrolysis conditions, different concentrations of acid were studied for varying periods of time; in the second stage, the temperature was varied with time, keeping the concentration constant in order to of minimize the factors of the study.

The data reported in table 3 show the behavior of the acid hydrolysis of ALB as a function of the H_2SO_4 concentration and hydrolysis time, while in table 4, the NEPP content is reported which was obtained varying temperature and time of acid hydrolysis (5% de H_2SO_4) on ALB.
The highest NEPP value (11.89±0.26 (GAE)/g dw) was found with 5% of H_2SO_4 at 80°C for 90 min of hydrolysis reaction in the first stage. Observing the hydrolysis behavior, it was decided to keep the concentration at 5% for the next study stage.

Table 3: NEPP obtained in the sulfuric acid hydrolysis of cell Wall-Bond of ALB (step 1)

H_2SO_4 (%v/v)	T (min)	TP 1 ((GAE)/g dw)	TP 2 ((GAE)/g dw)	TP 3 ((GAE)/g dw)	mean	SD
2.5	30	0.96	0.9	1.2	1.02	0.159
2.5	90	5.73	6.47	6.12	6.11	0.370
2.5	150	0.38	0.48	1.4	0.75	0.562
5	30	4.53	5.3	6.1	5.31	0.785
5	90	13.03	11.67	10.97	11.89	1.082
5	150	3.51	4.1	6.2	4.60	1.414
7.5	30	2.69	3.1	3.2	3.00	0.270
7.5	90	8.76	7.5	8.1	8.12	0.630

Temperature was 80°C; SD= standar deviation

Table 3 shows the NEPP content in the second acid hydrolysis stage. Here, it was observed that the maximum concentration of NEPP (40.72±1.93 (GAE)/g dw) was obtained at 110°C, and 90 min.

Table 4. NEPP obtained in the sulfuric acid hydrolysis of cell Wall-Bond of ALB (step 2)

T (°C)	T (min)	TP 1 ((GAE)/g dw)	TP 2 ((GAE)/g dw)	TP 3 ((GAE)/g dw)	mean	SD
90	30	5.72	4.26	5.16	5.05	0.737
90	90	15.73	13.15	11.24	13.37	2.253
90	150	8.17	7.95	6.66	7.59	0.816
110	30	12.15	11.38	12.16	11.90	0.447
110	90	38.6	41.2	42.37	40.72	1.930
110	150	14.99	15.68	15.33	15.33	0.345
130	30	2.43	3.7	2.89	3.01	0.643
130	90	21.63	26.14	19.25	22.34	3.499
130	150	6.75	10.15	8.97	8.62	1.726

SD= standar deviation

In figure 2a y 2b, the graphics of response for the output of polyphenols are shown according to H_2SO_4 concentration and the time of hydrolysis, as well as temperature versus time, respectively. The mathematical model that represents it (equation 3) allowed to obtain the conditions for optimization of the acid hydrolysis in the first stage (5% de H_2SO_4 during 89 minutes, to 80°C). In the second stage (111°C, during 90 minutes, 5% de H_2SO_4) the mathematical model was obtained (equation 4).

$$z = -19.18 + 0.298x + 6.57y - 0.0017x^2 - 0.00041xy - 0.62y^2 \quad (3)$$

(R^2=95.2%)

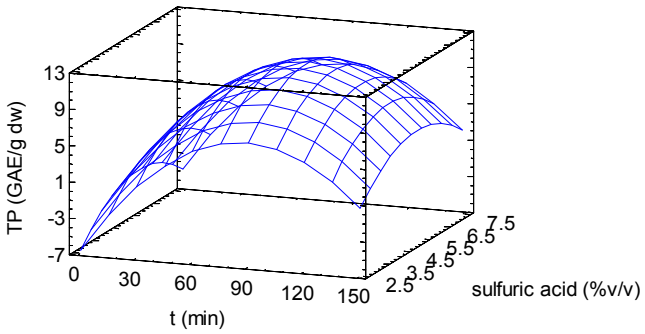
$$z = -390.7 + 0.80x + 6.97y - 0.0047x^2 - 0.00064xy - 0.032y^2 \quad (4)$$

(R^2=86.1%)

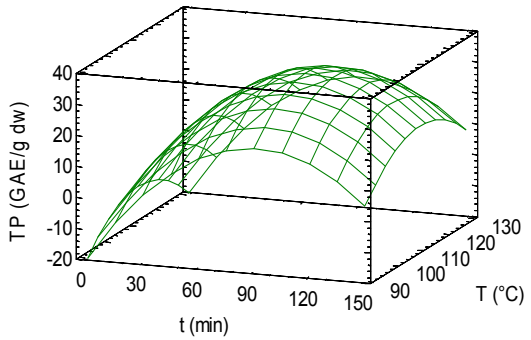
Z= total phenolics ((GAE)/g dw), x=time (min), y= sulfuric acid (% v/v)

From all the above, the effect that the concentration H^+ has on breaking bonds of the structure of lignin is observed (first stage) for the release of NEPP that is potentiated when the temperature is increased (second stage). This sort of compounds are not released by the action of organic solvents due to its molecular arrangement, which makes interaction with these compounds difficult, therefore the importance of finding the optimal conditions of hydrolysis when taking into account both effects.

This type of hydrolysis brings about the advantage of being both economical and easy to perform, compared to the enzymatic method that is commonly used to break down the biomass lignocellulosic structure[37].



a)



b)

Fig.2 Response surface model plot in terms of TP content, for hydrolysis of ALB, showing the effects of a) sulfuric acid and time; b) temperature and time

The NEPP content obtained at the optimization hydrolysis conditions (41, 2 (GAE)/g dw) is notoriously higher than the one reported by Conde *et al.* [22] for the hydrolysis of the sugar cane treated with 7% H_2SO_4, during 15 minutes at 121 °C.

It is clearly observed that there is a much more content of TP in the NEPP (to 18.66 times) in comparison to EPP (table 5) reported in fruits by Arranz *et al.*

When the potential fraction of NEPP is not released from biomass by hydrolysis and remains in the matrix of lignocellulose, it can be consumed by a human as dietetic fiber, which is deposited in the colon, where it begins the process of a poor hydrolysis by the colonic microbiota, having a low rate of release of NEPP. Yet, by releasing NEPP from the lignocellulose matrix in a previous step and consumed in a diet, one portion would be absorbed by the gastrointestinal tract and the pieces that reach the colon intact will contribute to the protector effect against the pro-oxidant substances as mentioned by Saura-Calixto[35].

3.4 Antioxidant activity of EP and NEP of ALB: In table 5, the IC_{50} of the different types of ALB is reported. It is observed that both the EPP and the NEPP are capable of inhibiting 50% of the radicals DPPH in a range of concentrations between 0.73 and 1.66 (GAE)/g dw. Although there is no statistical difference between EPP 1 and 2 in terms of TP content, the IC_{50} decreases by 50% for the latter. The AOxA measured

by the reduction of DPPH radical does not show correlation with the content of TP in different types of polyphenols.

Table 5**1. IC_{50} of DPPH• values of EPP and NEPP**

Poliphenol type	IC_{50} (GAE/g dw)	PT GAE/g dw
EPP 1	1.56[b]	6.9±0.31[a]
EPP 2	0.73 [a]	7.6±0.56[a]
NEPP 1E	0.97 [a]	11.89±0.26 [b]
NEPP 2E	1.66 [b]	40.72±1.93 [c]
EPP N	0.89	40.18
EPP M	1.58	21.88

EPP 1= EPP (28°C, 4h), EPP 2= EPP (85°C, 5min), NEPP 1E= NEPP of cell Wall-Bond (first stage), NEPP 2E= NEPP of cell Wall-Bond (second stage), EPP N= EPP of Navo [38], EPP M= EPP of Malva [38]. *Values not sharing common letters are significantly different (P<0.005)*

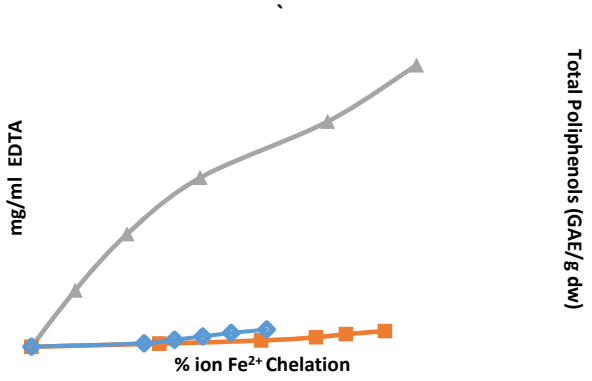


Figure 3. Chelation activity of EPP1 (■), EPP2 (◇) from LBCA compared to EDTA (▲)

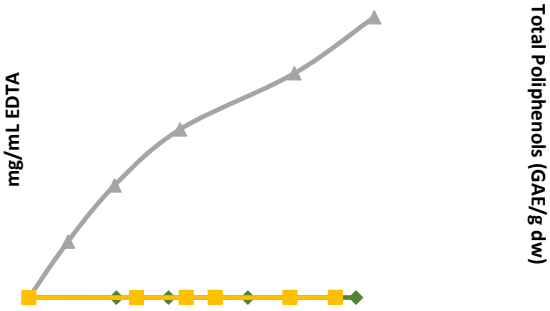


Figure 4. . Chelation activity of NEPP 1 (◇), NEPP 2 (■), from LBCA compared to EDTA (▲)

EPP 1= EPP (25°C, 4h), EPP 2= EPP (87°C, 5min), NEPP 1E= NEPP of cell Wall-Bond (step 1), NEPP 2E= NEPP of cell Wall-Bond (step 2)

The NEPP obtained from ALB present higher antioxidant activities than EPP; also NEPP have an AOxA similar to the organic extracts obtained from turnip and mallow (Gutierrez *et al.* [38], which are plants used as forage. However, further study employing different oxide-reduction systems must be performed to prove the efficiency of the antioxidants. By testing the effect of the antioxidants with the chelating power of the ion Fe^{2+}, it was observed that the EPP coming from ALB of *Amaranthus hypochondriacus* L. had a higher chelating power on the ion Fe^{2+} in comparison with the one reported by Ozsuy[39] who found a 73.4% of chelation for *A. lividus* in a range of 0-40mg/mL.

As for NEPP, evidence of chelating activity can be observed even at lower concentrations (in ppm), which indicates that these hydrolysates could be powerful chelating agents. Evaluation of the chelating capacity of polyphenols on metallic ions is highly important since metals are capable of starting lipoperoxidation reactions of unsaturated fatty acids. Thus, the use of NEPP hydrolysates represents a new option for the alimentary industry to avoid lipoperoxidation reactions, induced by the presence of Fe^{2+} ions.

CONCLUSIONS

Temperature and time are factors that affect the process of acid hydrolysis for obtaining ALB cell wall-bound compounds that present interesting AOxA. ALB is proposed in this research as a potential renewable source of antioxidant compounds.

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