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Antioxidant Activity of Fermented and Nonfermented Coffee (*Coffea arabica*) Pulp Extracts

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Summary

Coffee pulp contains natural antioxidants like hydroxycinnamic acids, most of which are covalently linked to the cell wall. These compounds can be released by fermentation or enzymatic processes. In this study, the antioxidant properties of fermented and nonfermented coffee pulp have been evaluated. Coffee pulp was fermented by solid-state fermentation using the fungus *Aspergillus tamarii*. Fermented and nonfermented samples of coffee pulp were extracted with aqueous methanol followed by alkaline hydrolysis. In both cases, the total polyphenol concentration was quantified by Folin-Ciocalteu method, then hydroxycinnamic acids were concentrated using ethyl acetate and quantified by HPLC. The antioxidant properties of samples were determined by radical monocation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) $[ABTS]^{\cdot+}$: the antioxidant activity was determined by kinetic parameters known as ED_{50}, $t_{ED_{50}}$ and antiradical efficiency (AE). Fermented extracts containing free hydroxycinnamic acids showed better antiradical activity against $[ABTS]^{\cdot+}$ than the other nonfermented ones. There were no significant differences in the total content of polyphenols in fermented and nonfermented coffee pulp, but the content of total hydroxycinnamic acids was higher in the nonfermented coffee pulp extracts (47.1 g/kg) than in the fermented coffee pulp (30.9 g/kg). Nevertheless, the fermentation process increased the fraction of free hydroxycinnamic acids (47 %) and consequently decreased those covalently linked to the cell wall. The results of the antioxidant activity assays could be explained by the presence of free hydroxycinnamic acids. Fermented coffee pulp assays showed that free hydroxycinnamic acids were metabolised by *A. tamarii*. This study shows the potential of using coffee pulp as a natural source of antioxidants.

Key words: antioxidants, hydroxycinnamic acids, $[ABTS]^{\cdot+}$, solid-state fermentation

Introduction

Coffee pulp (CP) has been largely used as a substrate for enzyme production. In the last years, hydroxycinnamic acids (HAs) have been recognised as natural antioxidants. HAs like chlorogenic (ChA), caffeic (CA) and ferulic acid (FA) are natural compounds in coffee pulp (*1,2*). They have only one aromatic ring and several hydroxyl groups (*3,4*). FA, CA and ChA are HAs present in CP with higher antioxidant properties. These compounds are quickly able

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Abbreviations: $[ABTS]^{\cdot+}$: radical monocation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid), AE: antiradical efficiency, CA: caffeic acid, ChA: chlorogenic acid, CP: coffee pulp, FA: ferulic acid, HAs: hydroxycinnamic acids, *p*-CA: *p*-coumaric acid, SSF: solid-state fermentation

to donate a hydrogen atom to an oxidized molecule or one in danger of transforming. The importance of an antioxidant depends on its concentration, the reaction medium and its ability to interact with regenerative systems (*5*). There are two ways of recovering free HAs from CP by a chemical or enzymatic process: (*i*) HAs are extracted with solvents of different polarity after alkaline hydrolysis to release HAs linked to the cell wall, and (*ii*) using enzymes (cinnamoyl esterases) to release HAs bound to the wall. In the latter case, the extracted HAs maintain the label of natural products.

The expression of antioxidant activity by kinetic parameters was done using the 2,2-diphenyl-picrylhydrazyl radical ($DPPH^{\cdot}$) assay by Brand-Willams *et al*. (*6*), and Sánchez-Moreno *et al.* (*7*). Recently, Pérez-Jiménez and Saura-Calixto (*8*) have applied this kinetic parameter approach to the $[ABTS]^{\cdot+}$ method, which is one of the more widely used ones. It is based on the scavenging ability of the antioxidants for the long-life radical cation $[ABTS]^{\cdot+}$. The preformed radical monocation is generated by oxidation of ABTS with potassium persulphate and is reduced in the presence of hydrogen-donating antioxidants. This scavenging produces a decrease in the absorbance reading at 734 nm. The reading of the absorbance as a function of reaction time of the radical and the presence of antioxidants are shown graphically. The area under the curve generated by the inhibition of the absorbance is calculated. The remanent $[ABTS]^{\cdot+}$ is obtained by interpolating from the Trolox standard plot. The aim of this study is to evaluate the antioxidant properties of the extracts from fermented and nonfermented CP. Fermented samples were obtained by cultivation of the fungus *Aspergillus tamarii.*

Materials and Methods

Fermentation of coffee pulp

CP from *Coffea arabica* was collected in Veracruz State, Mexico, at the end of the 2006 season after being sun dried. CP was fermented with *A. tamarii*. Solid-state fermentation (SSF) of CP was carried out in a stainless steel semi-pilot reactor (70 L) agitated by helicoidal ribbons, described in detail by Nava *et al.* (*9*). CP was previously sterilised (121 °C for 15 min) and inoculated with $2{\cdot}10^7$ spores per g of dried matter. For each 100 g of coffee pulp, the following compounds were added (in g): diammonium tartarate 6.15, yeast extract 1.7, KH_2PO_4 0.65, $CaCl_2{\cdot}2H_2O$ 0.045, $MgSO_4{\cdot}7H_2O$ 1.65 and maltose 8.35 (*10*). CP was incubated at 30 °C and aerated at the fixed rate of 1 L/(min·kg) of wet matter (*11,12*).

Hexane extraction

A mass of 10 g of CP was extracted with 50 mL of hexane at 30 °C, under agitation at 100 rpm for 30 min in Erlenmeyer flasks in order to eliminate carotenoids and waxes (*2*). All extracts were done in triplicate.

Determination of free HAs

Successive extractions with 50 mL of methanol/water 80:20 (by volume), acidified with 1 % acetic acid, were carried out at 55 °C with agitation at 100 rpm for 35 min. This process was repeated three times to get a final volume of 150 mL. In this extraction, HAs, polyphenols and soluble sugars were recovered from the CP (*2*). Methanol removal from the extracts was carried out under vacuum in order to concentrate polyphenols. Extracts were acidified to pH=3 with hydrochloric acid (J. T. Baker, Mexico City, Mexico) 50 % (by volume), pH=3 (*7*). Selective extractions with ethyl acetate were performed in a separating funnel; at a ratio of sample/ethyl acetate of 1:2 (by volume) allowing to separate the aqueous phase and the organic phase. Polyphenols were recovered in the organic phase (*13*). HAs extracted under these conditions were considered as HAs not linked (free) to the cell wall.

Determination of total polyphenols and covalently linked HAs

Insoluble material in aqueous methanol (10 g) was hydrolysed with 150 mL of 2 M NaOH at 45 °C under agitation of 100 rpm for 2 h (*13*). Samples were acidified with hydrochloric acid 50 % (by volume, pH=3) (*7*). Each sample was analysed for total polyphenolic content by a modified Folin-Ciocalteu method (*14*). HAs were analysed by HPLC as indicated below. Polyphenols and HAs recovered under these conditions were considered to be polyphenols and HAs covalently linked to the cell wall.

Estimation of antioxidant capacity

The method used to measure antioxidant capacity was a spectrophotometric method using $[ABTS]^{\cdot+}$, which is obtained after the reaction of [ABTS] (7 mM) with potassium persulphate (2.45 mM final concentration) incubated at room temperature (±25 °C) in darkness for 16 h. Once formed, the radical $[ABTS]^{\cdot+}$ is diluted with buffer (5 mM NaCl and 5 mM NaH_2PO_4) until an absorbance of 0.70±0.1 is obtained (*15*). Decrease of the absorbance was observed in the presence of antioxidants. Trolox was used as a standard, which is known to be an analogue of vitamin E and is used as a standard antioxidant.

Estimation of ED_{50} and $t_{ED_{50}}$

ED_{50} is defined as the concentration of antioxidant needed to reduce 50 % of the radical cation $[ABTS]^{\cdot+}$, concentration, $t_{ED_{50}}$ is the time required for the antioxidant to reduce the original concentration of radical cation $[ABTS]^{\cdot+}$ to 50 %. Kinetics was performed until the reaction time reached a steady state where there were no significant changes in the radical concentration (*8*). The assays were performed using a spectrophotometer at a wavelength of 734 nm for less than 30 min (*8*). The antiradical efficiency (AE) was expressed as follows:

$$AE=(ED_{50}\times t_{ED_{50}})^{-1} \qquad /1/$$

HPLC analysis

A sample of 10 μL of ethyl acetate extract was analysed on an HPLC system equipped with SPD-M20A array diode detector, a SIL-20AHT autosampler, a DGU-20A5 degasser and Lab Solutions software (all Shimadzu Scientific Instruments, Kyoto, Japan). Analyses were conducted at room temperature. Separation was performed using a Varian Polaris 5 amide C-18 (Agilent Technolo-

gies, Santa Clara, CA, USA) reversed phase column (200 ×4.6 mm).

HAs were analysed at 320 nm. A modification of the method described by Kim *et al.* (*16*) was as follows: mobile phase A: acetonitrile, and mobile phase B: acetic acid 2.5 % (by volume). Flow rate was 1.2 mL/min. The applied gradient was as follows: 0–10 % A for 30 min, 10–20 % A for 50 min, 20–30 % A for 55 min, 30–50 % A for 60 min, 50–0 % A for 65 min and then isocratic conditions were applied using 100 % B for 5 min.

Results and Discussion

The free and covalently linked phenolic compounds in fermented and nonfermented CP are shown in Table 1. No significant differences were found in the total content of phenolic compounds in fermented (322±30) g/kg and nonfermented (335±71) g/kg of CP. Fermentation reduces the linked phenolic compounds (63.6 to 57.3 %) and increases free phenolic compounds (36 to 42 %) in fermented and nonfermented CP but no significant differences were detected in these results.

Table 1. Total polyphenolic content of fermented and nonfermented coffee pulp

Extract	*w*/(g/kg)	
	Fermented CP	Nonfermented CP
Free polyphenols*	(137.8±23)[a]	(122±8)[a]
Covalently linked polyphenols**	(184.6±7.1)[b]	(213±63)[b]
Total	(322.4±30.1)[c]	(335 ±71)[c]

*extracted with aqueous methanol
**extracted after NaOH hydrolysis
Results are expressed as the mean value±standard deviation on dry matter basis
[a, b, c] the same letter indicates no significant difference ($p<0.05$)

In this sense, the content of free and linked HAs is presented in Table 2. Fermented CP presented a reduction (34 %) in total HAs in comparison with nonfermented CP. An important result was that the occurrence of esterified HAs in nonfermented CP was reduced from 86.8 to 53 % compared to the fermented CP. These changes can be explained by fungal growth. After the SSF with *A. tamarii*, the ratio of free HAs to those linked to the cell wall was changed (Table 2). In nonfermented CP, 13 % of total HAs were not linked (free) to the cell wall and after fermentation, free HAs (*p*-CA, FA, CA and ChA) increased to 47 % of the total content of phenolic compounds measured.

Table 2. Hydroxycinnamic acid content of fermented and nonfermented coffee pulp

	w/(g/kg)	*w*/(g/kg)
	Fermented	Unfermented
Free HAs*	(14.5±6)[b]	(6.2±1.05)[a]
Covalently linked HAs**	(16.4±1.3)[b]	(40.9±8.6)[c]
Total	(30.9±7.3)[c]	(47.1±9.65)[c]

*extracted with aqueous methanol and concentrated with ethyl acetate
**extracted after NaOH hydrolysis and concentrated with ethyl acetate
Results are expressed as the mean value±standard deviation on dry matter basis
[a, b, c] the same letter indicates no significant difference ($p<0.05$)

Antioxidant activities of the extracts from fermented and nonfermented coffee pulp were assayed by $ABTS^{\cdot+}$ and the kinetic results are plotted in Fig. 1. Comparison of the antioxidant capacity of the two materials is shown, where fermented CP is seen to have a higher antioxidant capacity than nonfermented CP.

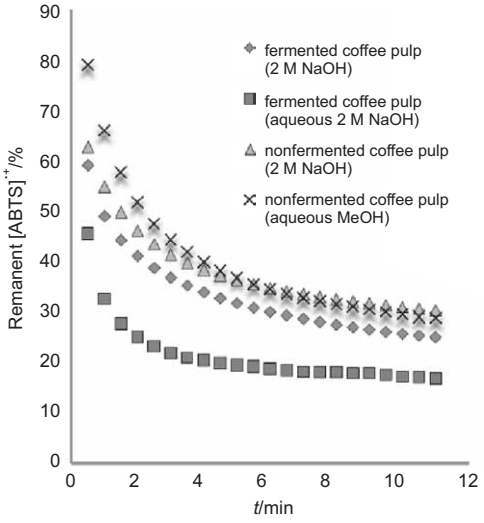

Fig. 1. Remanent $[ABTS]^{\cdot+}$ *vs.* time of different extracts from fermented and nonfermented

Polyphenols extracted with aqueous methanol from fermented CP were shown to have better antioxidant activity than other extracts obtained after alkaline hydrolysis and those obtained from nonfermented CP. This behaviour could be due to the presence of free HAs. Table 3 shows the kinetic parameters of the antioxidant capacity of fermented and nonfermented CP. The kinetic parameters, ED_{50} and $t_{ED_{50}}$ were calculated for the extracts of free and esterified phenolic acids from CP; the values of $t_{ED_{50}}$ less than 5 min were classified as antioxidants with a faster reaction rate (*7*), which was the case for all the assayed extracts.

Although no significant differences were noted in the total content of polyphenols in both materials, it was higher in the nonfermented CP. It is interesting to note that the fermented CP showed a slight increase (13 %) in the concentration of free polyphenols, as measured by the Folin-Ciocalteu method (Table 1). Nevertheless, an important increase in the proportion of free HAs was observed (134 %) in the case of fermented CP (Table 2). These changes are due to the enzymatic action of *Aspergillus tamarii* on the cell wall and they explain the antioxidant properties of fermented CP. HAs are probably released and then metabolised by the fungus. Metabolic activity of *A. tamarii* can also explain the increase in AE (Table 3).

Table 3. Kinetic parameters of fermented and nonfermented coffee pulp

Extract	Fermented			Unfermented		
	ED_{50}	$t_{ED_{50}}$	AE	ED_{50}	$t_{ED_{50}}$	AE
	mg	min	$mg^{-1}\cdot min^{-1}$	mg	min	$mg^{-1}\cdot min^{-1}$
Free polyphenols*	$(0.02\pm0.007)^a$	$(80.41\pm0.035)^a$	121951^d	$(0.19\pm0.087)^c$	$(1.2\pm0.002)^b$	4314^b
Covalently linked polyphenols**	$(0.034\pm0.004)^b$	$(1.12\pm0.19)^b$	25982^c	$(2.1\pm0.13)^d$	$(2.0\pm1)^b$	236^a

*concentrated with ethyl acetate after aqueous methanol extraction
**concentrated with ethyl acetate after NaOH hydrolysis
Results are expressed as the mean value±standard deviation
[a,b,c,d]the same letter indicates no significant difference ($p<0.05$) for ED_{50}, AE and $t_{ED_{50}}$

Table 4. HPLC analysis of hydroxycinnamic acid content in methanol extracts before and after ethyl acetate extraction of fermented and nonfermented CP

Extract	*w*(*p*-coumaric acid)	*w*(ferulic acid)	*w*(caffeic acid)	*w*(chlorogenic acid)
	mg/kg	mg/kg	mg/kg	mg/kg
		Unfermented coffee pulp		
Aqueous MeOH	$(4\pm0.02)^c$	$(1.5\pm1.4)^a$	$(100\pm0.27)^c$	$(140\pm0.7)^c$
Ethyl acetate	$(3.3\pm0.3)^b$	$(7.5\pm4.1)^c$	$(10\pm7.1)^b$	$(560\pm1.7)^d$
		Fermented coffee pulp		
Aqueous MeOH	$(4.4\pm0.003)^d$	$(1.5\pm0.2)^a$	$(8\pm0.003)^b$	$(5\pm4.7)^a$
Ethyl acetate	$(1\pm0.6)^a$	$(2.5\pm1)^b$	$(2.4\pm0.02)^a$	$(15\pm1.5)^b$

Results are expressed as the mean value±standard deviation
[a,b,c,d]the same letter indicates no significant difference ($p<0.05$) for each measured HA

Aqueous methanolic extracts were analysed by HPLC and compared with standards of ChA, FA, *p*-CA and CA. Table 4 shows the HA content before and after ethyl acetate extraction of the two studied materials. Results showed that ethyl acetate preferentially extracted ChA and FA, while aqueous methanol preferentially extracted *p*-CA and CA. These results should be taken into account for further research. ChA remained the most abundant HA in the extracts, as reported by other authors (*17*,*18*). Our research established the relationship between free HAs in CP and their antioxidant capacity and opened an interesting potential use of CP, a by-product of the coffee industry.

Conclusions

Coffee pulp could be a potential source of antioxidants. SSF of coffee pulp by *A. tamarii* does not significantly modify the total content of free and covalently linked phenolic compounds, as measured by the Folin-Ciocalteu method. Nevertheless, a large increase (134 %) in free hydroxycinnamic acids was observed in fermented CP in comparison with the nonfermented material, correlating with the antioxidant properties measured in this study. Antioxidant properties of fermented and nonfermented CP were evaluated by a kinetic approach as estimated by mean dose (ED_{50}), mean time ($t_{ED_{50}}$) and antiradical efficiency (AE). The best results of antioxidant capacity were found for the fermented extract containing the highest content of free HAs. Reduction of phenolic compounds in fermented CP was due to the solid-state culture of *A. tamarii*. These results are experimental evidence of the potential of using coffee pulp as a natural source of antioxidants which can be extracted through fermentative and enzymatic processes.

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