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Solid-State Fermentation as a Sustainable Tool for Extracting Phenolic Compounds from Cascalote Pods

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Abstract: Cascalote pods are an important source of phenolic compounds, mainly recovered using solvent extraction methods. As a sustainable alternative, this study aimed to evaluate solid-state fermentation (SSF) to enhance the extractability of total phenolic compounds (TPC) with antioxidant activity (AA) from cascalote pods. *Aspergillus niger* GH1 was selected based on the amount of TPC released and AA measured (ABTS, DPPH, FRAP) in a shorter time. Moreover, moisture, temperature, inoculum size, and mineral composition were evaluated. The largest amount of TPC released was 124.17 mg/gdm (g of dry material) after 12 h of culture, strongly and highly significantly correlated with the AA. Moisture and KH_2PO_4 concentration were the main influencing factors on TPC release. Treatment 6 [1x 10^7 spores/gdm, 30 °C, 60% moisture, mineral composition (g/L): KH_2PO_4, 1.52; $NaNO_3$, 7.65; and $MgSO_4$, 1.52] was selected due to the highest values of both TPC and AA. SSF-assisted extraction allowed increase 118 % and 93 % in TPC and AA values, respectively. Corilagin, lagerstannin, geraniin, and ellagic acid were the main phenolic compounds identified by RP-HPLC-ESI-MS in cascalote extracts. The results obtained demonstrate the feasibility of SSF-assisted extraction as a biotechnological alternative for the recovery of important bioactive molecules from this underutilized material.

Keywords: condensed tannins; hydrolysable tannins; ABTS; DPPH; solid-state fermentation; improved extraction; RP-HPLC-ESI-MS

1. Introduction

Caesalpinia coriaria is a plant found in the Pacific coastal plains of Central America, commonly called "cascalote" or "divi-divi", which is used to obtain firewood, charcoal, poles, and beams. This plant produces pods 3 to 7 cm long, with a green color when unripe and dark brown color when ripe. According to Palma García [1], one cascalote tree can produce up to 150 kg of pods, which indicates a large production.

These pods are commonly used in leather tanning and fodder and are traditionally used as a treatment for infectious skin problems [2]; however, most of the pods produced are underutilized and, consequently, not fully exploited. Also, economic activity derived from harvesting has declined. Cascalote pods are astringent due to their high content of phenolic compounds, important molecules with a great interest in health, mainly due to their biological activities, such as antimicrobial, antioxidant, and anti-inflammatory [3,4]. The main phenolic compounds reported in cascalote

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fractions with biological activity are gallic acid, ethyl gallate, stigmasterol, tannic acid, and corilagin [2,5].

Phenolic compounds are commonly recovered using solvents and several extraction techniques, such as maceration, microwave, and ultrasound, among others; however, their use involves expensive equipment, as well as environmental pollution and toxicological safety concerns regarding the use of solvents [6,7]. As a biotechnological alternative, the use of fermentative or enzymatic methods for assisted extraction of biomolecules from vegetal matrices has been reported, solid-state fermentation (SSF) included [8].

SSF does not require the use of solvents and promotes high yields and easy recovery of bioactive compounds. During the SSF process, polyphenols could be biosynthesized or biotransformed into simpler molecules using the activity of microbial enzymes [9,10], which degrade the wall cell components, increasing the extraction of free and bound polyphenols [11]. Recently, SSF has been successfully used to obtain bioactive compounds from rambutan peel [12], orange peel [13], pomegranate husk [14], and pineapple waste [15], among others; however, the use of SSF in cascalote pods as a substrate to obtain bioactive compounds has not been studied. Based on responsible production and consumption, this work aimed to develop an eco-friendly bioprocess, SSF, for the extraction, recovery, and identification of compounds with antioxidant capacity from cascalote pods.

2. Materials and methods

2.1. Raw material

Cascalote pods were collected in San Miguel Totolapan, Guerrero, México (18.152094485006664, -100.36604425470092) and transported to the laboratory of Biotechnology & Bioengineering in Delicias, Chihuahua, Mexico. They were dehydrated (50 °C, 24 h), pulverized (PULVEX Mini 100, Mexico City) to a particle size of 1 mm, and stored in a hermetic black polyethylene bottle at room temperature (30 °C) until use.

2.2. Physicochemical characterization of cascalote pods

Protein, fat, carbohydrate, fiber, moisture, and ash content were determined according to the Association of Official Agricultural Chemists (AOAC) procedures [16]. The critical humidity point (CHP) and water absorption capacity (WAC) were obtained according to Orzua, *et al.* [17]. The maximum material moisture was obtained using solids, moisture content, and WAC values [12].

2.3. Microorganisms

For this study, the strains *Aspergillus niger* GH1, *Aspergillus awamori,* and *Aspergillus niger HT4* (belonging to the DIA-UAdeC collection) were used. The GH1 strain of *A. niger* was deposited in the Micoteca of the University of Minho with the number MUM:23.16. Fungal strains were cryopreserved at -55 °C in a skim milk/glycerol (9:1 v/v) solution. Fungal spores were activated on potato dextrose agar (PDA-Bioxon™) plates at 30 °C for 5 days. For the inoculum preparation, fungal spores were collected with a sterile solution of Tween-80 (0.01 % v/v) and counted in a Neubauer chamber.

2.4. Solid-state fermentation (SSF)

Cascalote pods mixed with vermiculite (50:50 w/w) were used as support and the sole carbon source for fungal growth. SSF was performed in column reactors (31.5 x 180 mm) packed aseptically with a homogeneous mixture containing 6 g of fermentable mass with initial moisture of 50% (3 g of support impregnated with 3 mL of Czapeck-Dox saline solution, previously inoculated with 1 x 10^6 spores/g of support). A saline solution was prepared to contain (g/L): KCl (1.51), $MgSO_4$ (1.52), $NaNO_3$ (7.65) and K_2HPO_4 (3.04). SSF was monitored at 30 °C for 72 h, withdrawing samples every 12 h to determine the maximum phenolic content time. To obtain the fermented extracts, 12 mL of 50% ethanol was added to each reactor. Samples were shaken in a vortex and filtered through cotton and filter paper (0.45 μm) before being placed into 2 mL vials and stored at -18 °C until analysis.

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2.5. Evaluation of the SSF factors for the total polyphenol extraction

Once the fungal strain was selected, the maximum phenolic content time and antioxidant activity were selected as well. Other SSFs were conducted in parallel to identify the effects of inoculum, temperature, moisture, and the concentrations of KCl, $MgSO_4$, $NaNO_3$, and K_2HPO_4, according to Table 1. The extracts were obtained after 12 h of SSF with *A. niger* GH1 and used to determine the TPC and the activity in antioxidant assays.

Table 1. A Box, Hunter, and Hunter condensed matrix was used to determine the influence of independent factors (A, B, C, D, E, F, and G) on TPC and AA (ABTS, DPPH, FRAP) of extracts obtained by SSF with *A. niger* GH1 from cascalote pods.

Treatment	A	B	C	D	E	F	G	TPC (mg/gdm)	ABTS (mgTE/gdm)	DPPH (mgTE/gdm)	FRAP ($mgFE^{+2}$/gdm)
1	-1	-1	-1	1	1	1	-1	71.62 ± 2.23^{f}	271.46 ± 3.15^{de}	237.32 ± 5.51^{f}	735.78 ± 12.85^{d}
2	1	-1	-1	-1	-1	1	1	62.95 ± 3.51^{g}	247.90 ± 15.01^{e}	226.25 ± 7.39^{f}	737.49 ± 15.09^{d}
3	-1	1	-1	-1	1	-1	1	99.88 ± 1.96^{c}	380.68 ± 19.01^{b}	365.92 ± 4.54^{c}	945.92 ± 55.67^{c}
4	1	1	-1	1	-1	-1	-1	79.48 ± 0.52^{e}	294.85 ± 8.28^{cd}	297.58 ± 6.97^{e}	800.07 ± 53.24^{d}
5	-1	-1	1	1	-1	-1	1	92.55 ± 1.45^{d}	384.55 ± 15.28^{b}	345.96 ± 9.63^{cd}	925.42 ± 39.09^{c}
6	1	-1	1	-1	1	-1	-1	124.17 ± 1.94^{a}	447.64 ± 5.81^{a}	498.46 ± 13.15^{a}	1251.13 ± 29.45^{a}
7	-1	1	1	-1	-1	1	-1	114.23 ± 2.67^{b}	438.81 ± 14.00^{a}	452.64 ± 3.94^{b}	1121.07 ± 44.45^{b}
8	1	1	1	1	1	1	1	71.84 ± 2.78^{f}	320.19 ± 14.71^{c}	329.10 ± 15.32^{d}	1019 ± 52.78^{bc}

Code	Factor	Low level (-1)	High level (1)
A	Inoculum (spores/gdm)	$1x10^6$	$1x10^7$
B	Temperature (°C)	30	40
C	Moisture (%)	50	60
D	KH_2PO_4 (g/L)	1.52	3.04
E	$NaNO_3$ (g/L)	3.04	7.65
F	$MgSO_4 \cdot 7H_2O$ (g/L)	1.52	3.04
G	KCl (g/L)	1.52	3.04

*Different lowercase letters indicate no significant differences among treatments (Tukey's test; $p \leq 0.05$).

2.6. Analytical analysis

2.6.1. Determination of polyphenol content

The hydrolysable polyphenols (HP) assay was carried out according to Wong-Paz et al. [18]. In a 96-well microplate, 20 µL of the sample, 20 µL of Folin-Ciocalteu reagent (2 N), 20 µL of 20% sodium carbonate solution, and 125 µL of distilled water were mixed. After 5 min of incubation, the absorbance was measured at 790 nm in a microplate reader (Multiskan GO, Thermo Scientific). The results were expressed as mg of gallic acid equivalents/g of dry matter (mgGAE/gdm), using a calibration curve (0–1 mg/mL).

The condensed polyphenols (CP) were determined using ferric reagent and HCl-butanol (1:9 v/v) [12]. The sample (250 µL) was mixed with 1,500 µL of HCl-butanol, and 50 µL of ferric reagent in 20% HCl was added. The mixture was sealed hermetically and boiled for 40 min. Finally, the samples were cooled and 200 µL were placed in a 96-well microplate, and the absorbance was recorded at 460 nm using a microplate reader (Multiskan GO, Thermo Scientific). The results were expressed as mg of catechin equivalents/g of dry matter (mgCE/gdm), using a calibration curve (0–1 mg/mL). The TPC

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was determined by adding the values of hydrolysable and condensed polyphenols and expressed as mg/g of dry matter (mg/gdm).

2.6.2. Antioxidant activity

The antioxidant activity of the extracts was evaluated based on DPPH, ABTS, and FRAP assays. For the DPPH assay, 60 µM DPPH [2,2-diphenyl-1-picrylhydracyl (Sigma-Aldrich®, México)] stock solution (2.365 mg dissolved in 100 mL absolute ethanol) was prepared. The sample (7 µL) and DPPH stock solution (193 µL) were placed in a 96-well microplate, mixed, and kept in darkness for 30 min at room temperature [19]. A microplate reader (Multiskan GO, Thermo Scientific) was used to measure the absorbance at 517 nm.

The ABTS decolorization assay was carried out using the protocol adapted to a microplate [20,21]. The $ABTS^{\bullet+}$ was prepared by mixing 7 mM ABTS [2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate)] (Sigma-Aldrich®, México) solution (0.3602 g in 100 mL distilled water) with 2 mM potassium persulfate (2.45 µM, 0.0662 g in 100 mL distilled water). The mixture was kept in darkness for 12–16 h at room temperature. After that, the absorbance of $ABTS^{\bullet+}$ was adjusted to 0.70 at 734 nm. The sample (10 µL) and $ABTS^{\bullet+}$ (190 µL) were mixed, and after one minute, the absorbance was measured using a microplate reader (Multiskan GO, Thermo Scientific). The results for DPPH and ABTS assays were expressed as mg of Trolox equivalents/g of dry matter (mgTE/gdm), using a calibration curve with 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox; 0–1 mg/mL).

The FRAP (ferric reducing antioxidant power) assay was carried out according to Pulido, *et al.* [22], adapted to a 96-well microplate. The FRAP reagent [0.3 M acetate buffer (pH 3.6), 10 mM 2,4,6-tri(2-pyridyl)-s-triazine (TTPZ; Sigma-Aldrich®, México) solution in 10 mM HCl, and 20 mM $FeCl_3.6H_2O$; 10:1:1 v/v] was prepared daily and incubated at 37 °C for 30 min. For the reaction, 6 µL of sample, 18 µL of distilled water, and 180 µL of FRAP reagent were mixed. The resulting solution was incubated at 37 °C for 60 min, and the absorbance was measured using a microplate reader (Multiskan GO, Thermo Scientific) at 595 nm. The results obtained were expressed as mg of Fe^{+2} equivalents/g of dry matter ($mgFe^{+2}$/gdm), using a calibration curve with Fe_2SO_4 (Sigma-Aldrich®, México) in 80% methanol (0–1 mg/mL).

2.6.3. Identification of phenolic compounds

The phenolic profile of fermented extract was obtained by reverse phase-high performance liquid chromatography/electrospray ionization/mass spectrometry (RP-HPLC/ESI/MS) according to Buenrostro-Figueroa, Nevárez-Moorillón, Chávez-González, Sepúlveda, Ascacio-Valdés, Aguilar, Pedroza-Islas, Huerta-Ochoa and Arely Prado-Barragán [14], using a Varian HPLC system including an autosampler (Varian ProStar 410, USA), a PDA detector (Varian ProStar 330, USA), and a ternary pump (Varian ProStar 230I, USA). The separation was carried out at 30 °C in a Denali C18 column (150 mm × 2.1 mm, 3µm, Grace, USA) in samples of 5 µL. The mobile phase (wash phase) was methanol, and the eluents were formic acid (0.2 %, v/v) and acetonitrile (solvent A and B, respectively). A mass spectrometer (Varian 500-MS IT Mass Spectrometer, USA) equipped with an electrospray ionization source, operated in negative mode $[M-H]^{-1}$, was used for LC-ESI-MS analysis. 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 of 50–2000.

2.7. Experimental design

To select the best strain and time for the largest TPC content and AA, a completely randomized design with factorial arrangement was established. The factors evaluated were fungal strain (*A. niger* GH1, *A. awamori*, and *A. niger* HT4) and culture time (0, 12, 24, 36, 48, 60, and 72 h) in triplicate (N = 63). After that, a 2^k Box, Hunter, and Hunter (BHH) design was set up to identify the factors with a significant effect on TPC extraction and AA. For this purpose, six factors (inoculum size, temperature, moisture, $MgSO_4$, $NaNO_3$, and K_2HPO_4), at two levels each, were used to construct a condensed matrix with eight treatments (Table 1). All treatments were performed in triplicate and expressed as

a mean (n = 3) ± standard deviation. Data were submitted to Analysis of Variance (ANOVA) and Tukey´s test using Statistica 7.0 software (Stat Soft, Tulsa, OK, USA). A p-value ≤0.05 was considered to indicate a significant difference. Correlations between phenolic compounds content (HP, CP, and TPC) and the activity in antioxidant assays (ABTS, DPPH, and FRAP) were determined using Pearson´s correlation coefficient (r).

3. Results

3.1. Physicochemical characterization of cascalote pods

The chemical composition of cascalote pods (Table 2) was determined to evaluate their nutritional feasibility to be used as a substrate for fungal growth in a SSF process. The main components in cascalote pods were carbohydrates and fiber, whereas proteins constitute both elemental carbon and nitrogen sources for fungal growth and enzyme production [15]. The physicochemical values for water absorption capacity (WAC), critical humidity point (CHP), and maximum moisture of the substrate (see Table 2) indicate the potential of any material to be used as substrate in a SSF process.

Table 2. Physicochemical characterization of cascalote pods**.**

Parameter	Content
Moisture (g/100 gdb)	3.36 ± 0.15
Fat (g/100 gdb)	0.65 ± 0.09
Fiber (g/100 gdb)	6.54 ± 0.25
Protein (g/100 gdb)	3.44 ± 0.13
Ash (g/100 gdb)	2.13 ± 0.21
Carbohydrates (g/100 gdb)	87.24 ± 0.96
Water absorption capacity (g of gel/gdw)	2.97 ± 0.07
Critical humidity point (%)	3.75 ± 0.29
Maximum moisture of cascalote pods (%)	79.33 ± 2.08

gdb: gram of dry basis; gdw: gram of dry weight.

3.2. Kinetics of TPC extraction and AA using Aspergillus strains

The TPC release and AA assays (DPPH, ABTS, and FRAP), related to fermentation time, are shown in Figure 1. All of the evaluated strains could use cascalote pods as a substrate-support for their growth. TPC release was fast at the beginning, reaching a maximal value after 12 h of fermentation for all of the strains. At 12 h, the amount of TPC released was 70.22, 102.21, and 117.69 mgGAE/gdm for *A. niger* HT4, *A. awamori*, and *A. niger* GH1, respectively. After that time, TPC started to decrease, attaining a concentration of 55 mgGAE/gdm for all of the strains. Similar patterns were observed in the AA assays, DPPH (Figure 1b), ABTS (Figure 1c), and FRAP (Figure 1d), which achieved the largest numbers at 12 h for all of the strains. Based on the obtained results, *A. niger* and 12 h of fermentation time were selected for further studies.

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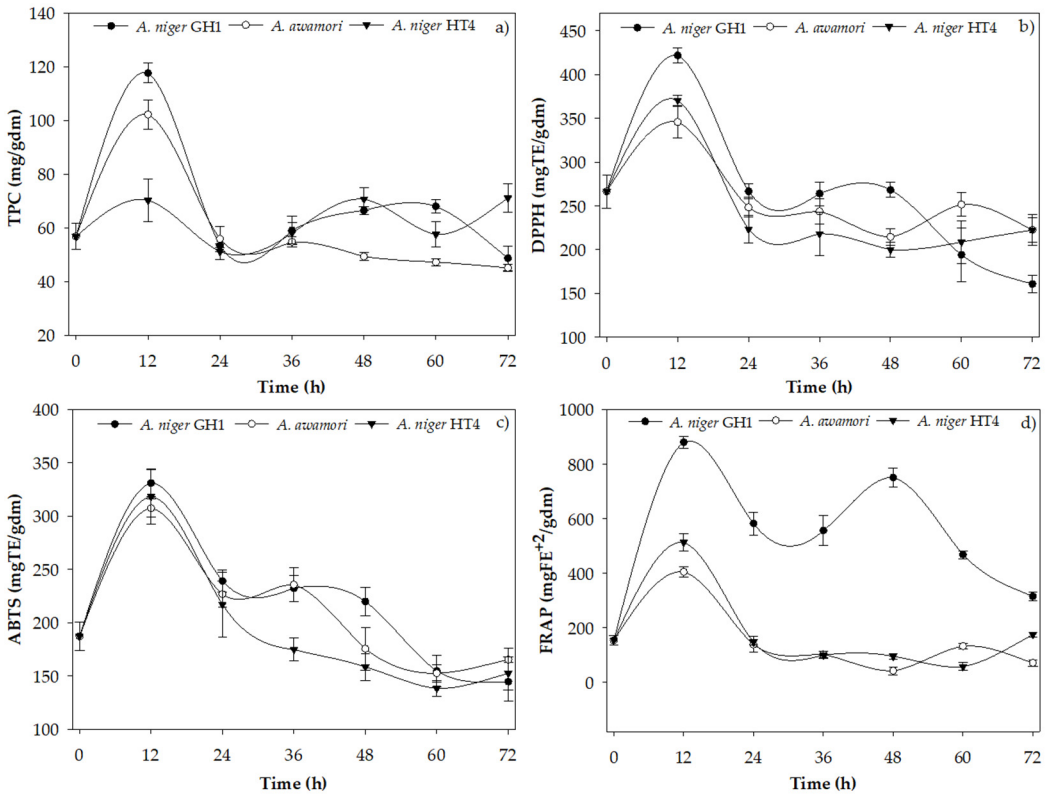

Figure 1. Kinetics of: a) total polyphenol content, b) antioxidant activity by DPPH assay, c) antioxidant activity by ABTS assay, and d) antioxidant activity by FRAP assay, for fermented extracts from cascalote pods by *Aspergillus* strains.

Data from the extraction kinetics were used to estimate the relation between the phenolic compounds and antioxidant activity of fermented extracts from cascalote pods through the Pearson correlation coefficient. As shown in Table 3, the phenolic compounds content (HP, CP, and TPC) was strongly positively and highly significantly correlated with activity in antioxidant assays (ABTS, DPPH, and FRAP).

Table 3. Pearson's correlation coefficients between phenolic compounds (HP, CP, and TPC) and activity in antioxidant assays (ABTS, DPPH and FRAP).

Variables	HP	CP	TPC	ABTS	DPPH	FRAP
HP	1	0.94*	0.98*	0.94*	0.94*	0.82*
CP		1	0.98*	0.93*	0.94*	0.84*
TPC			1	0.95*	0.95*	0.85*
ABTS				1	0.95*	0.87*
DPPH					1	0.95*
FRAP						1

*Correlation is significant at the 0.01 level.

3.3. Significant factors for TPC release by SSF

Experimental design is a useful tool to define the conditions for performing a minimal number of experiments. The BHH design was performed to identify the main factors that influence TPC release from cascalote pods by SSF. For that, an 8-treatment experimental matrix was constructed to evaluate three process parameters (inoculum size, temperature, and moisture) and three media components (KH_2PO_4, $NaNO_3$, and $MgSO_4$) on the content of hydrolyzed, condensed, and total

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polyphenols, as well as the antioxidant activity against DPPH and ABTS radicals in cascalote pods fermented with *A. niger* GH1 (Table 1). Maximal values were obtained in treatment 6, without significant differences with treatment 7, which were 0.98-, 0.79-, and 1.1-fold higher than those obtained in treatment 2 for TPC, ABTS, and DPPH values, respectively.

To evaluate the influence of these factors, absolute values of standardized effects were estimated, and their behaviors are shown in Figure 2. Moisture was the most influential ($p \leq 0.05$) with a positive effect, indicating that as the factor level increased, the expected response also increased (Figure 2a). In contrast, TPC release was negatively affected by KH_2PO_4, $MgSO_4$, and KCl concentrations (Figure 2b, 2c, and 2d), revealing that an increase in these factors harms the TPC release. Temperature and $NaNO_3$ showed no significant effect ($p \leq 0.05$). Therefore, any level evaluated for these factors could be used. Media components that show a negative (KH_2PO_4, $MgSO_4$, and KCl concentration) or non-significant (temperature and $NaNO_3$) effect are recommended to use in low levels (see Table 1). Therefore, treatment 6 was selected due to the highest values of TPC release and higher activity in antioxidant assays (DPPH, ABTS, and FRAP).

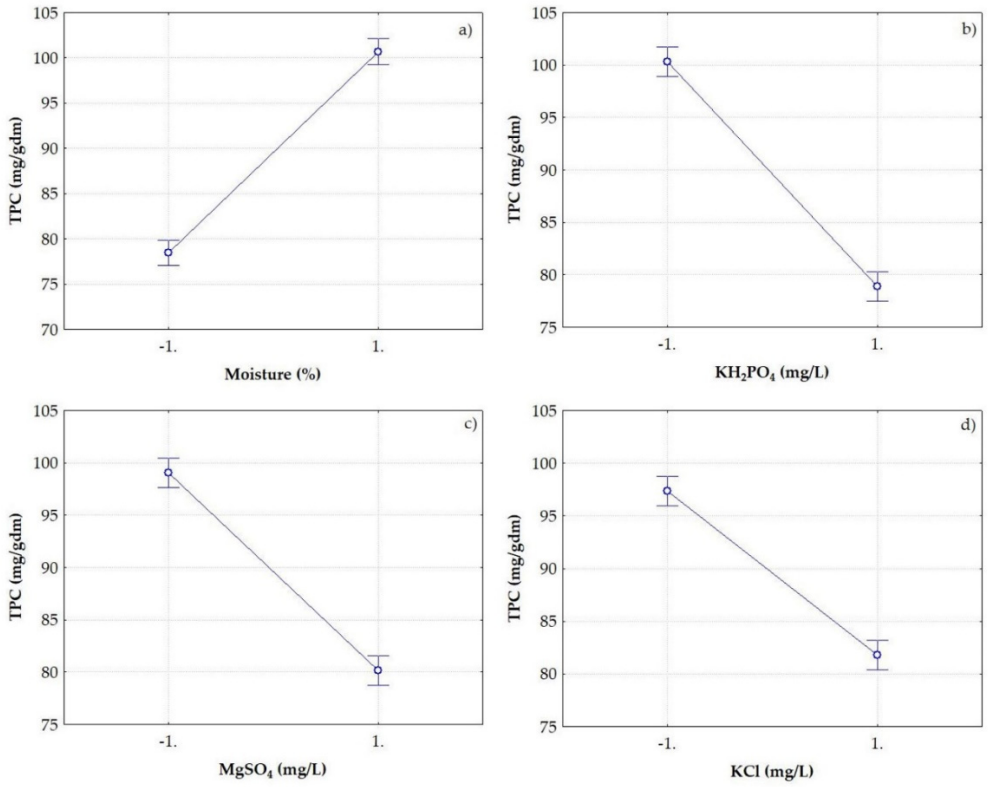

Figure 2. Effect of significant factors for TPC release by SSF with *A. niger* GH1.

3.4. Effect of SSF on chemical composition, tannin content, and antioxidant activity

Chemical composition, tannin content, and antioxidant activity of treatment 6 were determined, and compared to control, no fermentation was conducted (Table 4). Significant changes were observed for all of the parameters ($p \leq 0.05$), including an increase in moisture, fat, protein, and ash content. Reduction in fiber and carbohydrate contents by the effect of SSF was observed. Concerning tannin content, SSF by *A. niger* GH1 increased the content of hydrolyzed, condensed, and total polyphenols, reaching values 66%, 337%, and 118% higher than the control (without fermentation), respectively. The activity of fermented extracts in the DPPH, ABTS, and FRAP assays was improved by 66.76%, 93%, and 55.67%, respectively, compared to the control.

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Table 4. Effect of SSF of cascalote pods using *A. niger* GH1 on chemical composition, tannin content, and antioxidant activity.

Parameter/Treatment	Control (0 h)	Treatment 6 (12 h)*
Moisture (g/100 gdb)	4.90 ± 0.04^{b}	5.40 ± 0.20^{a}
Fat (g/100 gdb)	0.23 ± 0.05^{b}	0.34 ± 0.10^{a}
Fiber (g/100 gdb)	5.54 ± 0.23^{a}	1.77 ± 0.12^{b}
Protein (g/100 gdb)	3.24 ± 0.15^{b}	3.59 ± 0.10^{a}
Ash (g/100 gdb)	52.88 ± 0.95^{b}	59.28 ± 0.93^{a}
Carbohydrates (g/100 gdb)	38.11 ± 1.95^{a}	35.02 ± 1.76^{b}
Hydrolyzed polyphenols (mgGAE/gdm)	45.76^{b}	76.22^{a}
Condensed polyphenols (mgCE/gdm)	10.97^{b}	47.95^{a}
Total polyphenol content (mg/gdm)	56.73^{b}	124.17^{a}
Antioxidant activity:		
DPPH (mgTE/gdm)	266.63^{b}	444.64^{a}
ABTS (mgTE/gdm)	258.18^{b}	498.46^{a}
FRAP (mgFe^{+2}/gdm)	354.03^{b}	551.13^{a}

*Different letters indicated no significant differences between treatments (Tukey α=0.05).

3.5. Identification of phenolic compounds by RP-HPLC-ESI-MS

The extracts obtained at 0 and 12 h of SSF under treatment 6 conditions (see Table 1) were characterized by RP-HPLC-ESI-MS. A total of nine phenolic compounds were detected (Table 5), mainly belonging to the family of hydroxybenzoic acids and ellagitannins; however, two of them were unidentified according to the existing database, but according to the phenolic compounds profile and the weight of unidentified compounds, they are closely related to other ellagitannins. These compounds will be further elucidated using other procedures (i.e., Nuclear Magnetic Resonance).

Table 5. Phenolic compounds profile from cascalote pods fermented by *A. niger* GH1 and identified using the RP-HPLC-ESI-MS technique.

#	RT	$[M-H]^-$	Bioactive compound	Molecular formula	Family	0 h	12 h SSF
						UA	UA
1	10.41	342.5	5-O-Galloylquinic acid	$C_{14}H_{16}O_{10}$	Hydroxybenzoic acids	0.026	0.343
2	15.02	798.4	Ellagic acid derivate		Hydroxybenzoic acids	0.136	1.539
3	17.64	494.7	Unidentified			0.373	2.479
4	18.51	1118.1	Unidentified			0.384	2.451
5	20.51	632.6	Corilagin	$C_{27}H_{22}O_{18}$	Ellagitannins	0.747	2.501
6	24.18	782.4	Gallagyl-hexoside	$C_{34}H_{22}O_{22}$	Ellagitannins	0.338	ND
7	24.62	968.1	Lagerstannin B derivate		Ellagitannins	ND	2.458
8	25.79	952.2	Geraniin	$C_{41}H_{28}O_{27}$	Ellagitannins	0.377	2.307
9	28.84	300.6	Ellagic acid	$C_{14}H_6O_8$	Hydroxybenzoic acid dimers	0.247	2.155

RT = retention time; UA = unit of absorbance; ND = not detected.

4. Discussion

The culture media represents a mixture of nutrients that, in adequate concentrations and under optimal physical conditions, allows microbial growth and metabolic processes to occur [14]. The

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obtained results (Table 2) are in the range of values reported in the literature for moisture (3%), protein (3.85%–5.34%), ash (1.87%–2.58%), fat (0.19%–3.35%), and carbohydrates (71.62%–88.82%) contents [1,23].

In the SSF process, WAC, CHP, and maximum moisture are important parameters to evaluate the vegetal material's suitability as a solid support for fungal growth and metabolite production. WAC indicates the amount of water that can be absorbed by the substrate, related to the hydroxyl groups present on it, which allows additional water interactions by hydrogen bonding [12,24]. Thus, a high WAC value is convenient for the SSF process because moisture content can be modified, allowing microbial growth. Cascalote pods presented a WAC value of 2.97 g gel/gdw, which is in the range reported (2.16 to 3.4 g gel/gdw) for agro-industrial byproducts considered as suitable supports for SSF like grape waste [25], corn cobs [26], candelilla stalks [26], rambutan peels [12], and fig byproducts [27]. The CHP represents the water linked to the support, which cannot be used for the biological functions of the microorganism. The microbial growth is promoted at low CHP values, since high values affect it for the high proportion of water linked to the substrate, and therefore, free water content is low [15]. The CHP of cascalote pods was 3.75%, lower than those reported for fig byproducts (4.63%), sugarcane bagasse (9.46%), pomegranate peel (10.13%), coconut husk (16%), corn cobs (27%), grape waste (53.33%), pineapple waste (55.6%), and mango seeds (56.5%), which are materials successfully used as substrate-supports in SSF [11,14,15,25–27]. Additionally, cascalote pods could be used as substrate in SSF process at high moisture levels due to the maximum moisture obtained (79.33%); however, it is recommended to work at below 70% moisture to avoid oxygen transfer limitations, particle agglomeration, and bacterial growth [12,28]. Based on their physicochemical characterization, cascalote pods are suitable to be used as a substrate-support for SSF.

Filamentous fungi are the most used microorganism in SSF, and they have great potential to release bioactive compounds from vegetal matrices [8]. During SSF, fungi produce several enzymes that degrade the cell wall components, resulting in an enhanced phenolic compounds extraction [15]. *A niger* GH1 has been previously reported as a potential fungus for degrading lignocellulosic materials and releasing phenolic compounds [11,12,14]. In this study, the use of *A. niger* GH1 increased the TPC releasement 1.67-fold and the AA 2.17-fold compared to the other *Aspergillus* strains evaluated.

The Pearson correlation coefficient is a number between –1 and 1 that measures the strength and direction of the relationship between two variables. When one variable changes, the other variable changes as well but in the opposite or the same direction. The TPC releasement was strongly, positively, and highly correlated to AA (0.95; $p<0.01$), confirming that the increase in AA values is due to the increase in TPC release during the SSF process. This correlation is in accordance with the previous studies reported by Paz-Arteaga et al. [15], Jericó-Santos et al. [29], and Buenrostro-Figueroa et al. [14] in SSF of pineapple, tamarind, and pomegranate byproducts, which reported correlation values between TPC and DPPH from 0.63, 072, and 0.86, respectively. Increments in TPC released are associated with fungal enzymes produced during the microbial growth phase. These enzymes (xylanases, pectinases, proteases, and glucosidase, among others) are responsible for the oxidative degradation of lignin and the breakdown of the links between cell wall matrix and phenolic compounds, which results in their release [15,30].

Exploring the effects of nutritional and physical parameters in SSF promotes an increased TPC releasement. Moisture plays an important role in fungal growth. SSF requires a close control of water content; depending on the microbial strain used, a specific moisture content is needed to ensure growth and metabolite production [31]. In this study, TPC releasement was favored at high levels of moisture (60%), similar to the results obtained by Buenrostro-Figueroa et al. [27] in SSF of fig byproducts with *A. niger* GH1.

Magnesium is necessary for fungal nutrition, and it has an important role in hyphae development. An optimal concentration of this mineral improves the sporulation rate, promoting an efficient enzyme synthesis, and consequently, fungal biomass proliferation and phenolic compounds releasement [14,32]. Using *A. niger* GH1 and pomegranate husk to obtain ellagic acid and total

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phenolic compounds by SSF, Sepulveda et al. [32] and Buenrostro-Figueroa et al. [14] found that increments in $MgSO_4$ levels improved the releasement of ellagic acid and total phenolic compounds, respectively. Furthermore, those authors reported that the best phenolic compounds releasement was attained at high levels of KH_2PO_4 and KCl. In the present study, the same KH_2PO_4, KCl, and $MgSO_4$ levels were used; however, a contrary effect was observed. This may be due to the substrate-support (cascalote pods) itself containing sufficient amounts of minerals (Mg, P, and K) needed for both microbial growth and TPC releasement. The addition of higher amounts of minerals could affect the enzyme production [33]. The cell wall of *A. niger* contains carbohydrates, proteins, ash, and lipids [34]. Increases in protein and lipid content are related to the fungal biomass present in fermented cascalote pods. During fungal growth, the available nutrients are used to synthesize lipids as mycelium components. Reduction in carbohydrate and fiber contents are associated with fungal growth, since these components are used by the fungi as a carbon source for its development and production of cell wall-degrading enzymes [15,30].

SSF substantially improved the amount of phenolic compounds released from cascalote pods, as well as the antioxidant activity. This is explained by the fact that several enzymes with important hydrolytic activities participate in SSF, inducing the releasement of phenolic compounds from polymeric matrices [15,35].

Based on the above results, an SSF was successfully conducted by *A. niger* GH1 from cascalote pods, with 12 h as the best time for TPC releasement. This is the first report conducted for cascalote pods under the conditions described. Using extraction by maceration with methanol, Sánchez et al. [36] reported values of 21.71 mg/gmd of condensed tannins and 32.06 mg/gdm of total tannins. Rojas-Morales et al. [37] reported values for condensed and total tannins of 13 and 34 mg/gdm, respectively. A total condensed tannins content of 7.72 mg/gdm was reported by Pineda-Peña et al. [38]. The values for condensed and total polyphenols released from cascalote pods in the present work are 120%–521% and 265%–288% higher than the values previously reported [36–38].

In addition, cascalote pods exhibited strong antioxidant activity by reducing agents for ferric ions and scavenging free radicals, and SSF provided an increase of up to 93% in the AA of the extract (498.46 mgTE/gdm) compared to the value before SSF (258.18 mgTE/gdm), according to the ABTS assay. Ethanolic extract from cascalote was evaluated against DPPH and ABTS antioxidant assays [39]. At 500 mg/L, the inhibition rate was higher than 90% in both cases. Based on the strongly positive and highly significant correlation between phenolic compounds content and activity in antioxidant assays (DPPH, ABTS, and FRAP), the increase in AA is attributed to the amount and type of phenolic compounds released by the SSF. These results show the feasibility of SSF to obtain TPC with AA from vegetal matrices in comparison to chemical processes.

The RP-HPLC-ESI-MS of cascalote extracts showed that the main compounds were 5-O-galloylquinic acid, ellagic acid derivatives, corilagin, gallagyl-hexoside, lagerstannin B derivative, geraniin, and ellagic acid. There have been no reports indicating the identification of phenolic compounds obtained from cascalote pods by SSF; however, some of these compounds have been previously reported from hydroalcoholic extracts of *C. coriaria* [38,40]. In addition, other compounds, such as ethyl gallate, methyl gallate, gallic acid, tetragalloylglucose, pentagalloulglucose, valoneic acid dilactone, digalloylshikimic acid, and other phenolic compounds derivatives, have been cited [2,4,41].

From the nine compounds detected, seven of them increased in concentration at 12 h of SSF (in terms of units of absorbance), highlighting 5-*o*-galloyquinic acid, corilagin, geraniin, and ellagic acid with values 12.19-, 2.34-, 5.11-, and 7.72-fold higher than those obtained at 0 h of SSF. Lagerstannin B derivative was only found at 12 h of SSF, while gallagyl-hexoside was only detected at 0 h. The differences between the phenolic profile and absorbance values might be due to the phenolic compounds being in free or conjugated form (esterified). Therefore, during SSF these bonds are broken by the action of microbial enzymes, facilitating the partial or complete release of the phenolic compounds, improving their solubility or producing new molecules [15].

Identified polyphenol molecules in cascalote pods have different biological activities, such as antioxidant effects, hepatoprotective effects [39], anthelmintic effects [37,42], arginase inhibitory

activity [43], antimicrobial activity [4,41,44], and gastroprotective effects [38]. These benefits provide a wide range of possible applications in the food, pharmaceutical, and cosmetic industries.

Accurate data related to cascalote production was not found; however, a yield of 150 kg of pods per tree has been reported [1]. Using the described bioprocess here, up to 18.6 kg of TPC per tree of cascalote pods could be obtained (124 kg of TPC/ton). Considering the commercial prices of corilagin, geraniin, and ellagic acid (885 USD/10 mg, 877 USD/10 mg, and 420 USD/50 mg, respectively; Sigma-Aldrich®), the SSF extraction process represents a profitable and sustainable alternative to obtain valuable compounds with industrial applications. These results confirm that SSF permits the recovery of larger amounts of high-value molecules in a shorter time process. Focusing on the highly desired circular economy model, an alternative to the diversification of cascalote pods is reported.

5. Conclusions

A. niger GH1 utilized cascalote pods as a carbon source and released phenolic compounds with antioxidant activity. The factors that most influenced the TPC releasement were moisture and KH_2PO_4 concentration. The higher TPC releasement and antioxidant activity were obtained at the following SSF conditions: *A. niger* GH1 ($1x10^7$ sp/gdm) at 12 h of culture, 30 °C, 60% moisture, and a concentration of mineral salts in the medium (g/L) of: KH_2PO_4: 1.52, $NaNO_3$: 7.65, $MgSO_4 \bullet 7H_2O$: 1.52, and KCl: 1.52. Corilagin, lagerstannin, geraniin, and ellagic acid were the main phenolic compounds identified by RP-HPLC-ESI-MS in cascalote extracts. The development of a bioprocess like the SSF-assisted extraction conducted in this study allowed the significant increase of TPC releasement, exhibiting high AA, offering an alternative use for this underutilized material, cascalote pods.

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