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Antimicrobial activity of fermented *Theobroma cacao* pod husk extract

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ABSTRACT. *Theobroma cacao* L. contains more than 500 different chemical compounds some of which have been traditionally used for their antioxidant, anti-carcinogenic, immunomodulatory, vasodilatory, analgesic, and antimicrobial activities. Spontaneous aerobic fermentation of cacao husks yields a crude husk extract (CHE) with antimicrobial activity. CHE was fractioned by solvent partition with polar solvent extraction or by silica gel chromatography and a total of 12 sub-fractions were analyzed for chemical composition and bioactivity. CHE was effective against the yeast *Saccharomyces cerevisiae* and the basidiomycete *Moniliophthora perniciosa*. Antibacterial activity was determined using 6 strains: *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Bacillus subtilis* (Gram-positive) and *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Salmonella choleraesuis* (Gram-negative). At doses up to 10 mg/mL, CHE was not effective

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against the Gram-positive bacteria tested but against medically important *P. aeruginosa* and *S. choleraesuis* with a minimum inhibitory concentration (MIC) of 5.0 mg/mL. Sub-fractions varied widely in activity and strongest antibacterial activity was seen with CHE8 against *S. choleraesuis* (MIC of 1.0 mg/mL) and CHE9 against *S. epidermidis* (MIC of 2.5 mg/mL). All bioactive CHE fractions contained phenols, steroids, or terpenes, but no saponins. Fraction CHE9 contained flavonoids, phenolics, steroids, and terpenes, amino acids, and alkaloids, while CHE12 had the same compounds but lacked flavonoids.

Key words: Pod husk extract; *Theobroma cacao*; Antibacterial activity

INTRODUCTION

Cacao (*Theobroma cacao* L.) is a neotropical species native to the humid tropical plains of Central and South America (Whitkus et al., 1998). It is classified in the genus *Theobroma*, family Malvaceae esterculiacea and is considered to be a prehistoric tree already cultivated more than 3000 years ago by the Olmecs and Mayans (Dillinger et al., 2000). The Mesoamerican people used the seeds of *T. cacao* as bargaining chips and to produce beverages to be consumed during rituals or as a medicine to relieve symptoms associated with sicknesses of cardiovascular, gastrointestinal, and nervous nature (Keen et al., 2005; Henderson et al., 2007; Prufer and Hurst, 2007; Trognitz et al., 2011). Indigenous people from Central and South America still use cocoa in folk medicine (Jenny et al., 2009).

Cocoa has become an important ethnomedicinal plant since it has a unique chemical composition of more than 500 different compounds (Crown and Hurst, 2009). Among their reported contribution for human health, there are the antioxidant (Jalil and Ismail, 2008), antiinflammatory (Selmi et al., 2008), anticarcinogenic (Fotsis et al., 1997; Maskarinec, 2009), immunomodulatory, vasodilatory and analgesic (Wollgast and Anklam, 2000), and antimicrobial (Osawa et al., 1990; Percival et al., 2006; Summa et al., 2008; Fapohunda and Afolayan, 2012) activities.

Almonds or cocoa beans constitute the raw material for the production of chocolate and its derivatives, which are used in different forms worldwide. Processing of cocoa on the farms begins with the opening of the fruit (pods) in the field under the trees, where the seeds are separated from the husks. The seeds are then fermented in wooden boxes and sun-dried, to be later processed in the factory (Forsyth and Quesnel, 1957; Kalvatchev et al., 1998; Camu et al., 2008).

The cultivation of cocoa is of economic importance for several countries such as Ghana, Ivory Coast, Nigeria, Indonesia, Malaysia, and Brazil (Azizah et al., 2007; Hii et al., 2009). At the same time, commercial cocoa culture may represent a serious problem due to the generation of huge amounts of pod husks, unused organic material generally left behind in the fields. For each ton of dry seed about 7 tons of fresh pod husks are produced (Figueira et al., 1993). These residues, left in the field of most farms, may serve as reservoir of nutrients for various microorganisms (Vriesmann et al., 2012), including those responsible for different diseases of cacao, e.g., for the fungus causing witches' broom. Thus, by helping the propagation of unwanted fungi, uncontrolled deposits of cacao pod husks may lead to a reduction in cacao production (Kalvatchev et al., 1998). On the other hand, these cacao husks contain a range of biologically active compounds (Rice-Evans, 2001; Lecumberri et al., 2007) and, therefore,

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may represent a valuable source for the isolation of substances of economic value.

Here, we show that controlled spontaneous aerobic fermentation of cacao husks represents an economic alternative to their uncontrolled waste disposal and that this process not only decreases the extent of environmental problems but also yields an extract enriched with secondary metabolic compounds (such as phenols, alkaloids, terpenoids) with antimicrobial activity.

MATERIAL AND METHODS

Plant material

T. cacao plants selected for this study were Scavina-6 clones resistant to the fungus *Moniliophthora perniciosa* [formerly *Crinipellis perniciosa* (Stahel) Singer] growing in experimental areas of the Comissão Executiva de Planejamento da Lavoura Cacaueira-CEPLAC in Ilhéus, Bahia. The majority of cacao pods were harvested and processed at CEPLAC, while a 200-kg batch of pod husks was obtained from a farm not using chemical products (organic cultivar), serving as a control. After seed removal from the opened pods, the remaining husks were collected and transported to the Centro de Pesquisas do Cacau-CEPEC, Ilhéus, Bahia.

Preparation of cacao pod husk extract

Crude extract was prepared at CEPEC as described. Initially, 2 tons of cacao husks (from CEPLAC) were transferred to CEPEC (Figure 1A). To prepare CHE empty pod husks of *T. cacao* were chopped into rectangular pieces about 1.5 to 2.5 cm wide, slightly moistened, and placed on a polyethylene tarp to form piles 2.5 m long x 1.5 m wide x 1.0 m high (Figure 1B). During a 3-month period, the piles of chopped husks were stirred every 15 d until there was no more heat dissipation. Each pile was then transferred to a wooden container with a bottom sieve (4 mm mesh); 5 L distilled water was poured over the fermented husks, and the aqueous crude extract was collected in a bottom tray (Figure 1C). The crude extract obtained was transferred to glass Petri dishes (150 mm diameter), dried at room temperature for 72 h, ground in an electric grinder and passed through a 1.5 mm mesh sieve. Fourteen tons of fresh cocoa husks yielded 20 kg dry crude extract (Figure 1D). Pod husks from the organic cultivar were treated separately (as control) in the same manner.

Two grams of this crude extract were reduced to powder using a porcelain mortar, suspended/dissolved in 10 mL distilled water and filtered through a paper filter (Whatman, 80 g/m^2, porosity 3 μm). The remaining non-dissolved material was re-submitted to the same procedure twice to yield a total of 30 mL water-dissolved extract. The remaining insoluble residue was resuspended in 5 mL distilled water and subjected to sonication (Ultrasonic processor Gex 130, 130 W), with 10 pulses of 40 s each, 60% output with 10-s intervals, and filtered as described above. A total of 35 mL of this aqueous extract was frozen at -20°C, later concentrated by freeze-drying for 4 days to obtain CHE (yield of 72.95% w/w). A 100-g aliquot of CHE was subjected to standard microbiological (Downes and Ito, 2001) analysis.

Fractionation of CHE

CHE (100 mg) was fractionated by solvent partition by extraction with chloroform

(3 x 30 mL) (CHE1), ethyl acetate (3 x 30 mL) (CHE2), and n-butanol (3 x 30 mL) (CHE3), and the remainder was the distilled water fraction (CHE4). Other extractions were performed after hydrolysis, where 100 mg CHE were dissolved in 1 N sulfuric acid (5 mL). The mixture was refluxed for 30 min, filtered and alkalized to pH 10, yielding CHE5. This CHE5 fraction was submitted to solvent partition by extraction with chloroform/methanol (3:1 v/v), yielding CHE6 or chloroform (3 x 30 mL) yielding CHE7.

CHE (1.5 g) was chromatographed in an open column of silica gel 60RP-18 (a 40-63 Fluka Analytical), in the ratio 1:50 (extract:silica). Mixtures of methanol and water, 0 to 100%, were used as eluent. Forty-five fractions were obtained, which were grouped according to their chromatographic profile in thin layer chromatography (TLC) using silica gel 60 GF_{254} plates and n-butanol:acetic acid:water - BAW (4:1:1, v/v) as eluent, and visualized under UV light (254 and 365 nm, handheld UV Lamp Model 9403E, BioAmerica Inc., USA), resulting in five fractions (CHE8 to CHE12).

All fractions were dried in a rotary evaporator and stored at -20°C until used in biological tests. Yield for CHE and CHE-fractions are described in Table 1.

Antibacterial bioassay

The bacterial cultures were grown in Mueller-Hinton agar at 25°C for 24 h, thereafter suspended in 10 mL saline (0.9% NaCl) and suspensions adjusted to optical density for a 0.5 McFarland standard [10^8 colony forming units (CFU)/mL]. Six bacterial strains were used: a) Gram-positive *Staphylococcus aureus* (ATCC25921), *Staphylococcus epidermidis* (ATCC35984), and *Bacillus subtilis*, b) Gram-negative *Pseudomonas aeruginosa* (ATCC27853), *Klebsiella pneumoniae*, and *Salmonella choleraesuis*. Antibacterial bioassays using CHE or CHE-fractions were performed using micro-dilution broth with modifications (Basri et al., 2012). On a 96-well polystyrene microplate, we added to each well 80 µL inoculum (1.5 x 10^5 CFU/mL), 40 µL tryptic soy broth (TSB), and 80 µL of either CHE (original concentrations of 1.0, 5.0, and 10.0 mg/mL) or CHE-fractions (CHE1 to CHE12, at concentrations of 0.1-10 mg/mL). Negative control was the inoculum plus TSB and water instead of the CHE or CHE-fractions. Plates were closed and incubated at 37°C for 24 h. All tests were performed in triplicate. CHE and CHE-fractions were dissolved in distilled water. Antibacterial activity was assessed by optical density readings at 600 nm (Beckman DU-70 UV-vis spectrophotometer) at 0 and 24 h after inoculation. Minimum inhibitory concentration (MIC) of CHE was taken as the lowest concentration of the test agent that restricted growth increase to a level of OD < 0.05 of the control after the incubation period. For the results of the MIC the percentage of inhibition was calculated according to (Zampini et al., 2005). Results represent data of a minimum of 3 repetitions.

Antifungal bioassay

Two fungal species, *Saccharomyces cerevisiae* (BY4741, EUROSCARF) and *Moniliophthora perniciosa* were used in this study. *S. cerevisiae* was obtained by inoculation of an isolated colony into liquid YPD (1% yeast extract, 2% peptone, 2% glucose, 48 h). Survival of stationary yeast culture exposed to CHE (50-200 mg/mL, 1 h at 30°C in saline) was determined by plating appropriately diluted aliquots on solid YPD (triplicate) and incubating at

30°C for 2-3 days. *M. perniciosa* cultures were prepared by inoculation of aliquots during 7 days in CPD medium [2% peptone, glucose and agar, according to Filho et al. (2006)]. From those, 5 mL culture were incubated at different concentrations of CHE (50-200 mg/mL, 24 h). One milliliter of broken hyphae suspension was applied to three plates of CPD, and counted after 7 days, at 25°C. All results (yeast and *M. perniciosa*) were expressed as the percentage of survival related to the untreated controls and are the mean of at least three independent experiments; with error bars representing standard deviation as calculated by the GraphPad Prism program (GraphPad Software Inc., San Diego, CA, USA).

Phytochemical screening of CHE and bioactive CHE fractions

Contents of phytochemicals in CHE extracts were determined using 1 mg/mL of the respective extract in distilled water. The extracts were screened for the following classes of constituents: alkaloids (using both the Wagner and Mayer tests); anthraquinones (using the Bornträger test); cardiac glycosides (using the Baljet test); phenolic compounds (using $FeCl_3$ as the reagent); and saponins (using the Froth test) for their ability to produce suds (Harborne, 1998). The preliminary phytochemical analysis was also done for bioactive CHE fractions using silica gel 60 GF_{254} TLC plates and BAW (4:1:1, v/v) as eluent. The spots were first visualized under UV light (254 and 365 nm), then by specific reagents including 1% vanillin ammonia and 2% ferric chloride (polyphenolic compounds), or flavonoids (Natural Reagent -polyethylene glycol - NP/PEG), or anisaldehyde/sulfuric acid followed by heating (steroids and terpenes) or 0.1% ninhydrin (amines and amino acids). All these tests, procedures, and reagents are described elsewhere (Harborne, 1984; Wagner and Bladt, 1996).

Results and Discussion

Since little information is available on the presence of antimicrobial compounds in cacao pod husk, an extract was prepared (CHE) and its antimicrobial activity investigated (Figure 1). Dried CHE powder was black, and after dilution in water, it showed a dark-red color, absorbing at λ_{max} = 210 and 265 nm; at concentrations >1 mg/mL, it was a viscous liquid. The extraction process to obtain CHE yielded approximately 72.95% of the initial crude extract.

The antibacterial activity including growth inhibition and MIC assays by CHE and bioactive CHE-fractions are given in Tables 1 and 2, while antifungal activity of CHE tested with *S. cerevisiae* and *M. perniciosa* is shown in Figure 2. In Table 1, we can see that for *K. pneumonia*, there was a dose-dependent activity, i.e., 10X higher concentration led to 10X less bacterial growth. Strongest inhibitory activity was obtained against *P. aeruginosa* and *S. choleraesuis*. Interestingly, the growth of *P. aeruginosa* was only inhibited at 5 mg/mL CHE but not at the higher exposure dose of 10.0 mg/mL (Table 1). This puzzling result was obtained in each of 3 independent experiments and only with *P. aeruginosa*. The observed recovery of growth ability by this bacterium at higher dose of CHE might be explained by the presence of a compound in CHE that, at 10 mg/mL, can compensate for the original antibacterial activity witnessed at lower CHE doses, by inducing alteration in the bacterium's physiology. This compound could be either the same one responsible for the observed antimicrobial effect at lower doses (induction only at CHE doses >5 mg/mL) or another non-toxic compound of CHE that induces these changes in the bacterial metabolism. Similar observations were already reported by Saito et al. (2012) for the same bacterium, i.e., when testing antimicrobial activity of a plant extract (*Cassia alata*) in *P. aeruginosa*.

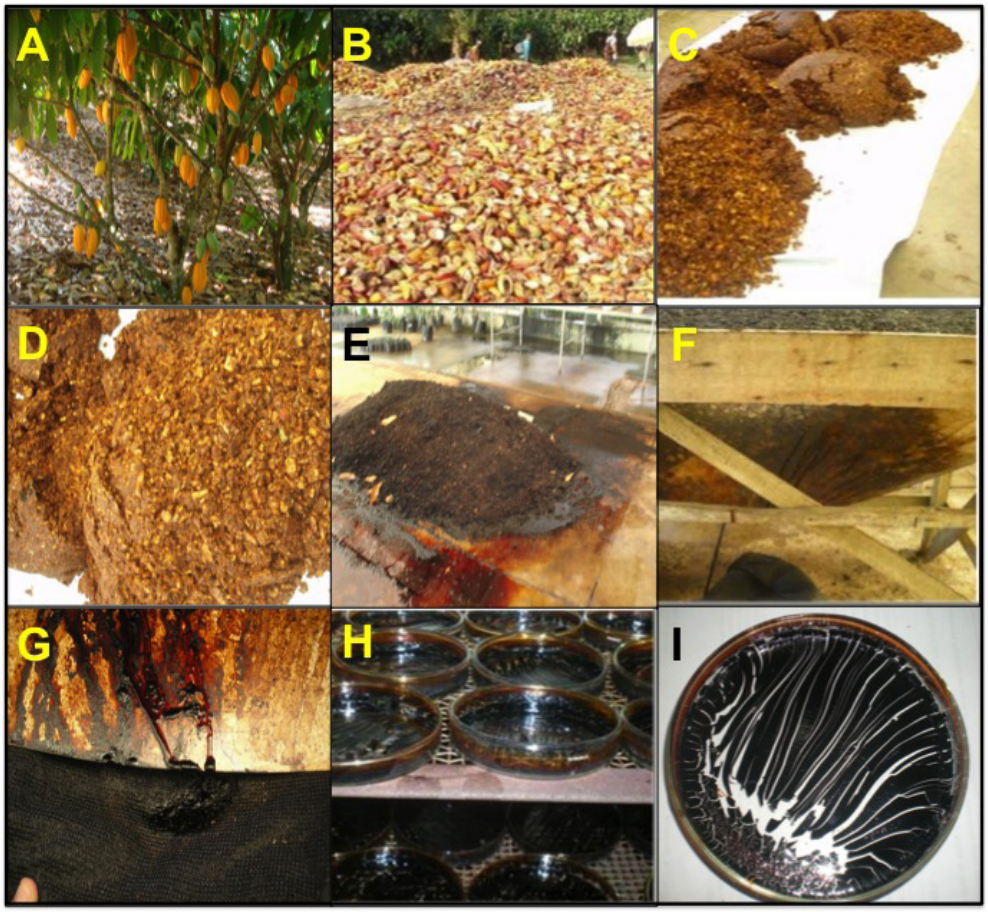

Figure 1. Various steps in the production of cacao husk extract (CHE): **A.** *Theobroma cacao* plant; **B.** Husks of *T. cacao* at CEPLAC farm; **C.-D.** piled chopped cacao husks, slightly humidified; **E.** chopped cacao husks after 60 days; **F.** chopped cacao husks in wooden recipient with a bottom sieve, to collect crude extract **(G)**; **H.-I.** dried crude extract obtained from husks of *T. cacao*.

Table 1. Bacterial growth inhibition by crude husk estract.

Microbial strains	Bacterial growth (%)		
	1.0 mg/mL	5.0 mg/mL	10.0 mg/mL
Gram-positive			
Bacillus subtilis	113.5 (± 6.6)	109.8 (± 3.5)	100.9 (± 1.7)
Staphylococcus aureus	134.1 (± 3.1)	144.5 (± 2.7)	103.2 (± 1.3)
S. epidermidis	100.9 (± 2.7)	98.3 (± 0.9)	92.5 (± 1.4)
Gram-negative			
Klebsiella pneumoniae	34.9 (± 3.6)	27.1 (± 4.2)	3.0 (± 0.2)
Pseudomonas aeruginosa	37.0 (± 3.6)	0.1 (± 2.1)	29.4 (± 9.4)
Salmonella choleraesuis	19.7 (± 5.2)	0.1 (± 8.8)	0.1 (± 1.1)

Data are reported as means ± SD.

Table 2. Chemical yields and inhibition of bacterial growth by crude husk extract-fractions.

CHE fractions	Chemical characteristic		Minimum inibitory concentration (mg/mL)		
	Extraction solvent (v:v)	Yield (%)	*Pseudomonas aeruginosa*	*Salmonella choleraesuis*	*Staphylococcus epidermidis*
CHE1	Chloroform	1.24		nt	
CHE2	Ethylacetate	0.19		nt	
CHE3	n-Butanol	0.27		>10	
CHE4	Water	59.80		>10	
CHE5	Water	61.55			
CHE6	Chloroform:ethanol (1:1)	0.39			
CHE7	Chloroform	0.21			
CHE8	Water	43.33		1.0	>10
CHE9	Water:methanol (7:3)	5.34	>10		2.5
CHE10	Water:methanol (5:5)	1.07			
CHE11	Water:methanol (3:7)	1.74			
CHE12	Methanol	0.21		1.0	>10

*Maximal applied dose was 10 mg/mL; nt = not tested. If no number is given, no activity was observed.

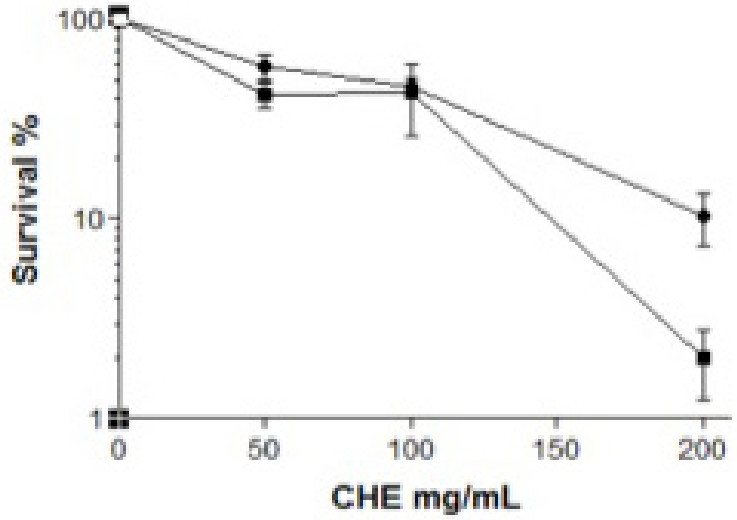

Figure 2. Survival of *Moniliophthora perniciosa* and *Saccharomyces cerevisisae* after crude husk extract (CHE) treatment. (●) *S. cerevisiae*, 1 h exposure; (■) *M. perniciosa*, 24 h exposure.

At doses of 1 to 10 mg/mL, CHE was not effective against any of the Gram-positive bacteria tested, but displayed bactericidal activity of varying degree against Gram-negative bacteria (Table 1). Gram-positive and -negative bacteria are commonly distinguished by their cell wall characteristics, which are related to the surface elements and the permeability properties. The probable chemical compound or group of compounds responsible for the specific bactericidal action of CHE in Gram-negative bacteria may have been extracted by the polar solvent (water). The outer membrane surrounding the cell wall of Gram-negative bacteria is known to contribute to the diffusion of hydrophilic compounds through its channel proteins, especially porins with hydrophilic characteristics (Tortora et al., 2010). This structural composition of the cell wall may contribute to the entry of bioactive compounds of increasing

polarity, i.e., CHE, which may have resulted in the observed increased cell death (Table 1).

While there were varying results between different bacteria, both yeast and *M. perniciosa* showed dose-dependent survival when exposed to CHE for 1 and 24 h, respectively (Figure 2). The data indicate that CHE had considerable *in vitro* activity not only against bacteria but also against the two fungi tested, since it inhibited the growth of both fungi in a dose-dependent manner. Broken hyphae of *M. perniciosa* proved to be significantly more resistant to CHE than yeast. The CHE-resistance of *M. perniciosa* resembled the much higher resistance to UVC when compared to yeast and also suggests that CHE does not induce oxidative stress damage, since *M. perniciosa* was found to be much more sensitive to H_2O_2 and paraquat as likewise treated yeast cells (Filho et al., 2006).

The production of the CHE antifungal activity during decay of the cacao husks is an aerobic process as treating the husk fragments in closed 60-L containers under anaerobic conditions for three months did not produce at any time effluents with antimicrobial activity (data not shown). Indeed, microbiological analysis of CHE showed the presence of aerobic psychrotrophic microbes (4.6×10^4 CFU/g), spores from thermophilic acidophilus bacteria ($>6.5 \times 10^3$ CFU/g) and mesophilic aerobic bacteria ($>6.5 \times 10^4$ CFU/g), and indicated the presence of thermophilic anaerobes, producers and non-producers of H_2S, and mesophilic aerobic bacteria. Also, no exothermic fermentation was observed, since daily measurements never yielded temperatures above 31°C, i.e., temperatures only 2 to 3 degrees above those of the environment. Also, bioactive compounds are unlikely to be proteins since boiling of CHE did not eliminate antifungal activity (data not shown). CHE derived from husks from organic cacao culture had likewise antimicrobial activity as the standard CHE derived from cacao husks from CEPLAC (data not shown), indicating that biotransformation of applied agrotoxic substances are not the source of the antimicrobial potential observed.

Various methods and solvents were used to obtain 12 CHE fractions and to identify whether the bioactivity observed depended on a single or a group of chemical compounds (Table 2). From those 12 fractions only CHE8, CHE9, and CHE12 were effective against two bacteria: Gram-negative *S. choleraesuis*, with a MIC of 1.0 mg/mL and Gram-positive *S. epidermidis*, with MIC of 2.5 mg/mL, a bacterium causing the majority of hospital infections (Table 2). According to Fabry et al. (1998), chemical compounds/extracts with MIC values less than 8 mg/mL are potentially useful candidates for therapeutic application. Therefore, the chemical components of CHE may be considered good candidates against Gram-negative bacteria of medical importance, such as *P. aeruginosa* and *S. choleraesuis*. The other fractions exhibited MIC above 10 mg/mL (CHE3 and CHE4 against *S. choleraesuis*) or undetectable activity (data not shown).

We observed strongest inhibitory activity of the CHE extract against *P. aeruginosa* (Table 1). When separating compounds present in CHE, one would expect that some CHE fraction produced even better inhibition against *P. aeruginosa*, because of the higher purity of the inhibitory compound obtained by fractionation (Table 2). However, our analysis showed that none of the fractions tested were active against *P. aeruginosa*. That could have been due to the existence of a "synergistic interaction" between substances present in CHE, which together could exert antibacterial activity against *P. aeruginosa* [synergism is characterized when the interaction of two or more compounds yields higher efficiency than either single compound (Varel, 2002)], or perhaps the active compounds were not eluted, but retained in the column.

Some information on the nature of possible antimicrobial substances in CHE and its sub-fractions (Table 2) was obtained. Better yields were obtained for water fractions indepen-

dently of method used (CHE4, CHE5, CHE8). Apparently, acidic hydrolysis of CHE rendered the extract without inhibitory power against any of the bacteria tested (CHE5, CHE6, CHE7). Fractions CHE4, CHE5, and CHE8 had a deep red color with smooth texture and showed yields of 59.80, 61.55, and 43.33%, respectively.

The physical characteristics of fractions CHE1, CHE2, CHE3, CHE6, and CHE7 obtained by different methods, showed ivory color, liquid texture and yielded extracts of less than 1.3% that could not inhibit bacterial growth, demonstrating that the majority of active compounds were polar ones.

In attempt to correlate the antibacterial activity with a chemical class of compounds, CHE was subjected to different colorimetric assays (Table 3), while the bioactive CHE-fractions were subjected to TLC for qualitative screening of the secondary metabolites (Table 2). The presence of phenolic compounds, steroids, or terpenes and absence of saponins was observed in all bioactive CHE-fractions. Fractions CHE8 and CHE9 were positive for flavonoids. Fractions CHE9 and CHE12 were positive for steroids, terpenes, and alkaloids (Table 3).

Table 3. Phytochemical screening of CHE and bioactive CHE-fractions.

	Extract	Bioactive fractions		
	CHE	CHE8	CHE9	CH12
Alkaloids	+	-	+	+
Amino acids	nt	-	-	-
Anthraquinones	-	nt	nt	nt
Cardiac glycosides	-	nt	nt	nt
Flavonoids	nt	+	+	-
Phenolic compounds	+	+	+	+
Saponins	-	-	-	-
Steroids and terpenes	nt	+	+	+

CHE = crude husk extract; (+) = presence; (-) = absence; nt = not tested.

The phytoconstituents in CHE and its bioactive CHE-fractions can be associated with their antimicrobial activity. Thus, the antibacterial activity observed (Table 2) cannot be attributed to only one class of phytochemicals (Table 3). The antimicrobial activities shown by the extract will likely be due to one or more of the several phytochemical constituents present in the extract. The phytochemicals detected during chemical screening are known as substances with important antimicrobial activity (Kumar et al., 2009). Some of these compounds, the terpenes, polyphenols, and alkaloids are known to have antibacterial, fungicidal, and insecticidal activity (Hernández et al., 2000; Akiyama et al., 2001; Ahmad and Beg, 2001).

CONCLUSION

Utilization of the enormous amounts of normally wasted cacao pod husks via fermentation and bioprocessing of their extract [CHE] may be a positive way to transform what could be an environmental problem into a biotechnological solution. Our study revealed that after spontaneous aerobic fermentation of cacao pod husks, the CHE obtained is an important source of metabolites containing bioactive compounds against bacteria of medical relevance, especially against Gram-negative *P. aeruginosa* and *S. choleraesuis*. CHE also revealed fungicidal activity against the basidiomycete fungus *M. perniciosa* and the yeast *S. cerevisiae*. CHE

contains a mixture of secondary metabolites either from the pod husks of *T. cacao* themselves or from bio-transformed derivatives formed during their aerobic fermentation or from the combination of both sources, as shown by the identification and presence of mainly phenolics and some nitrogen-containing compounds. Our results provide evidence that some phytochemicals extracted from aerobically fermented cocoa pod husks are bioactive and thus a potential source of natural antibacterial and antifungal agents. These results warrant further studies regarding analysis of the active CHE constituents and to determine their potential for therapeutic application.

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