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Use of *Theobroma cacao* Pod Husk-Derived Biofertilizer is Safe as it Poses neither Ecological nor Human Health Risks

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Abstract

Cacao farmers use macerated *Theobroma cacao* pod husks as biofertilizer for restoration of environmentally important soil elements, i.e., N, P, K, Na, Mg, and Ca. The increasing popularity of this organic material for soil management justifies to experimentally ascertaining that its application does not produce environmental or human health risks. We therefore applied a battery of biological tests to assess possible health risks related to the extract of fermented cacao pod husks [CHE]. Minimal inhibitory concentration for selected bacteria and fungi was established and an antiviral assay (equine herpes virus – EHV-1) and insecticide assay (*Aedes aegypti* larvae), to observe possible environmental impact. Toxicity tests used *Artemia salina*, hemolytic activity and cytotoxicity were tested using HT-29 e Vero cells, respectively. Genotoxicity and anti-genotoxic activity was tested in the comet assay of leukocytes and a selection of *Saccharomyces cerevisiae* mutants allowed look for a possible interference of CHE with defined metabolic pathways. None of these established tests used to define toxicity and genotoxicity of chemical compounds indicated that CHE contained substances that would pose such risks. Our safety assessment on bacteria and yeasts, virus, insect larvae, and human leukocytes [3-(4,5-di-methylazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) and comet assay] thus indicated that macerated cocoa pod husk can be safely used as biofertilizer.

Keywords: Cocoa pod husk; Fertilizer; Biosecurity

Introduction

The cocoa tree (*Theobroma cacao* L., Malvaceae) was domesticated approximately 3,000 years ago in Central America, the Caribbean, Africa and Asia [1]. Dried *T. cacao* seeds [beans] are the raw material for the food industry, mainly for chocolates, for pharmaceutical and cosmetic products. With an estimated value of annual production of some three billion dollars it is considered one of the main tropical crops worldwide. Unfortunately, cocoa production is negatively affected due to errors in soil management as well as by fungal diseases [2,3].

The intensity of soil use, typical for monoculture, leads to leaching of nutrients [4]. Research findings reported that a crop of 1,000 kg dry cocoa beans [obtained from approximately 20,000 fruits] removed about 20 kg nitrogen, 4 kg phosphorus and 10 kg potassium from the soil [5]. Without application of fertilizers, soils providing continuous production will thus lack essential plant nutrients. The high cost of industrially provided fertilizers led to intensive research for low-cost production of adequate organic material (biofertilizers). These should be preferentially locally produced, of non-toxic nature, and environmentally friendly. Such features would guarantee low-cost fertilizers for organic cocoa production [4].

Therefore, a most practical approach was to use the large quantities of cocoa pod husks, estimated at 800,000 tons annually, as an organic source for fertilizer production. Pod husk-derived fertilizer increases N, P, K, Ca, Mg, micronutrients and the pH status of soil and its correct application can thus partially or fully substitute the use of industrial fertilizers [6-14].

Although the positive effects of the application of cacao pod husk as fertilizer are already known for some time no study has yet been reported that would demonstrate the safety of its regular use as biofertilizer, especially regarding its potential to cause unwanted environmental impacts and problems in relation to human and animal health. We, therefore, aimed to assess if application of Cocoa Pod Husk-derived Extract (CHE), a concentrate of what would eventually leach from macerated pod husks, had the potential to negatively affect the environment and the human health. To this end we submitted CHE to a battery of standard toxicity and genotoxicity tests to ascertain its biosafety.

Materials and Methods

Plant material

Theobroma cacao plants selected for this study were Scavina-6 clone's resistant to the fungus *Moniliophthora perniciosa* [formerly *Crinipellis perniciosa* (Stahel) Singer] growing in experimental areas of the Executive Committee of the Cocoa Crop Planning (CEPLAC) in Ileus, Bahia. Most 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 for pest management (organic cultivar), serving as control. After seed removal from the opened pods, the remaining husks were collected and transported to the Cocoa´s Research Center (CEPEC), Ileus, Bahia.

Preparation of cacao pod husk extract

Crude husk extract (CHE) was prepared by chopping 2 tons of cacao pod husks (from CEPLAC), into rectangular pieces about 1.5 to 2.5 cm

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wide and, after slight moistening, placing them on a polyethylene tarp to form piles 2.5 m long × 1.5 m wide × 1.0 m high. During a 3-month period, these piles were stirred every two weeks 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. This crude extract was then 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. Pod husks from an organic cultivar (as control) were treated separately in the same manner [2].

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 3,000 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 Gex130, 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 analysis after dissolved in distilled water [15]. The physico-chemical characteristics of CHE are described in the patent entitled "PRIVILÉGIO DE INOVAÇÃO - EXTRATO DA CASCA DO FRUTO DO CACAUEIRO: OBTENÇÃO E USO AGRÍCOLA", number BR1120120171810 (source: www.inpi.gov.br/).

Minimum Inhibitory Concentration (MIC) of CHE

Antibacterial activity: Bacterial cultures were grown in Mueller-Hinton agar at 25°C for 24 h. There-after inoculum was suspended in 10 mL saline (0.8% NaCl) solution and adjusted to 0.5 McFarland (10^8 colony forming units (CFU)/mL). The bacteria used were: a) *Acidovorax avenae* pv. *Citrlii (*Aac1.12); *Pectobacterium carotovorum* pv. *Carotovorum* (Pcc23); *Ralstonia solanacearum* (CGH12); *Ralstonia solanacearum* - second strain (B19); *Xanthomonas campetris* pv. *Campetris* (Xcc56); *Xanthomonas campetris* pv. *Viticola* (Xcv112) (Table 1). All bacteria tested in the current study are gram-negative and of environmental importance - phytopathogenic and were kindly supplied by the Cultures Collection of the Laboratório de Fitopatologia from Universidade Federal Rural de Pernambuco (UFRPE).

Isolated code	Etiologic Agent	Type of cell wall	Plant Host	Habitat
Aac1.12	*Acidovorax avenae subsp. Citrlii*	Gram-negative	Yellow melon	Fruit
Pcc23	*Pectobacterium carotovorum subsp. carotovorum*	Gram-negative	Smooth lettuce	Leaf
CGH12	*Ralstonia solanacearum*	Gram-negative	Pepper	Branch
B19	*Ralstonia solanacearum (Second strain)*	Gram-negative	Banana silver	Fruit
Xcc56	*Xanthomonas campetris pv. campetris*	Gram-negative	Cabbage	Leaf
Xcv112	*Xanthomonas campetris pv. viticola*	Gram-negative	Vine	Branch

Table 1: Phytopathogenic bacteria strains used.

Antibacterial bioassays using CHE were performed using micro-dilution broth in a 96-well polystyrene microplate. Each well received 10 µL inoculum (1.5×10^5 CFU/mL), 100 µL tryptic soy broth (TSB), and 100 µL of CHE (final concentrations of 0.023, 0.045, 0.09, 0.18, 0.37, 0.75, 1.5 and 3.0 mg/mL). Plates were incubated at 25°C for 24 h. All tests were performed in triplicate [16-18].

Antibacterial activity was assessed using 30 µL of Rezasurin (Sigma-Aldrich, Brazil) to a 0.01% final concentration, 24 h after inoculation. Minimum inhibitory concentration (MIC) of CHE was taken as the lowest concentration of the test agent that restricted growth increase after the incubation period. Pink colour wells were considered positive and purple color wells were considered negative. Results represent data of a minimum of 3 repetitions.

Antifungicidal assay: Yeast cultures were grown in Sabouraud agar at 30°C (*Saccharomyces cerevisiae*) or at 37°C (*Candida sp.*) for 24 h, thereafter suspended in 10 mL saline (0.9% NaCl) and suspensions adjusted to optical density for a 1.0 scale McFarland standard [10^8 colony forming units (CFU)/mL] [17]. Five yeast strains were used: *S. cerevisiae* (ATCC 2691); *C. parapsilosis* (ATCC 22018); *C. krusei* (ATCC 6258); *C. glabrata* (ATCC 2301) and *C. albicans* (ATCC 14057).

Antifungal bioassay using CHE was performed using micro-dilution broth, with modifications. Using a 96-well polystyrene microplate, each well received 10 µL inoculum (1.5×10^5 CFU/mL), 100 µL yeast medium (YEL), and 100 µL of CHE (final concentrations of 0.023, 0.045, 0.09, 0.18, 0.37, 0.75, 1.5 and 3.0 mg/mL). Negative control contained the inoculum plus YEL and sterile water [16,19].

Plates were closed and incubated at 30°C (*Saccharomyces cerevisiae*) or at 37°C (*Candida sp.*) for 48 h. All tests were performed in triplicate. CHE was dissolved in distilled water. Antifungal activity was assessed using 2,3,5-Triphenyltetrazolium chloride (Sigma-Aldrich, Brazil), 48 h after inoculation. Minimum inhibitory concentration (MIC) of CHE was taken as the lowest concentration of the test agent that restricted growth increase. Results represent data of a minimum of 3 repetitions.

Antiviral assay

Vero cell lines (Vero, ATCC-CL 81) were used to perform antiviral assay [20]. Cells were maintained in MEM (Vitrocell/*Embriolife*®, Atená) supplemented with 8% of fetal bovine serum (FBS, Vitrocell/*Embriolife*®, Atená), with 5% CO_2 atmosphere at 37°C. Cells were seeded in a 96 well plate at 3×10^4 cells/well. After 24 h of incubation cells were treated with different concentrations of CHE (from 0.015 mg/mL to 4 mg/mL) and equine herpes virus (EHV-1) at 100 MOI and CHE at the same concentration and incubated at 5% CO_2 atmosphere at 37°C. After 48 h, the supernatant was removed and the 3-(4,5-dimethylazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, Brazil) mitochondrial reduction assay was performed. The CC50 (cytotoxic concentration for 50% of the cell culture) and IC50 (infectious concentration for 50% of the cell culture) were calculated. The selective index was obtained by the ratio between the extract CC50 and extract-virus IC50 (SI=CC50/IC50), which expresses the safety index for a determined test substance [21].

The Vero Cell line from green monkey kidney (ATCC CCL-81) was kindly provided by Instituto Biológico de São Paulo.

Preliminary evaluation of larvicidal activity against *Aedes aegypti*

The larvicide test was performed at the Laboratório de Pesquisa de Inseticidas Naturais (LAPIN), the Universidade Estadual do Sudoeste da Bahia (UESB), Itapetinga, Bahia [22,23]. The established colony of *A. aegypti* originated from eggs of Rockefeller lineage. The preliminary insecticide assessment of CHE on larvae of *A. aegypti* was conducted in controlled temperature (27.2°C) and room humidity (54.7%). Thirty larvae of *A. aegypti* third instar were evaluated, by repetition, totalling 150 larvae per treatment. Mortality was observed at 0, 1, 2, 4, 8, 16 and 24 h after starting the experiment. Larval mortality was characterized by absence of movement and staying at the container bottom, even when stimulated by a brush.

The insecticide potential of CHE was evaluated for five concentrations (0.75; 1.5; 3; 6 and 12 mg/mL). CHE was dissolved in a solution containing dimethylsulfoxide (DMSO) and deionized water in proportion of 1:9. This solution was also used to prepare the control group. The test was realized in quintuplicate.

Toxicity test using *Artemia salina*

CHE toxicity on the microcrustacean *Artemia salina* was tested according to [24]. A 24-well plate was seeded with 10 units of *A. salina* per well. Then CHE at eight different concentrations (0.046 mg/mL to 6 mg/mL) was added. Viability of CHE-exposed *A. salina* was observed after 0, 1, 2, 4, 8, 16, 24 and 48 h. Mortality is defined when there is no movement and larvae stayed at the container bottom, even when stimulated by a brush. A 0.2% solution of potassium dichromate in 3.5% saline served as positive control. Negative control was growth of *A. salina* without CHE. The test was performed in triplicate.

Hemolytic activity

Hemolytic activity was evaluated as described previously by [25], with slight modification by [26]. A suspension of human erythrocytes was washed with phosphate buffered saline (PBS: 1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 135 mM NaCl, pH 7.4) and then centrifuged at 6,000 × *g* for 10 min at least three times, until the supernatant was colorless. The human erythrocytes were then resuspended and diluted in PBS to 10 times the original volume to yield the stock erythrocyte suspension. Subsequently, 1.5, 3 and 6 mg/mL of water-dissolved CHE were incubated with 1 mL of human erythrocyte suspension (final erythrocyte concentration, 2% v/v) for 60 min at 37°C. Then intact erythrocytes were removed by centrifugation at 1.000 × *g* for 10 min. The released hemoglobin was then diluted 10-fold and the OD540 nm of the supernatant measured at. Hemolytic activity was calculated by use of the following formula: (Hemolysis %=Abs. of the sample-Abs. NC × 100/Abs. PC-Abs. NC) [27].

CHE cytotoxicity in eukaryotic cells – Cell Culture

HT29 cells and Vero cells were maintained in tissue-culture flasks at 37°C in a humidified atmosphere containing 5% CO_2, and were harvested by treatment with 0.15% trypsin and 0.08% of EDTA PBS. Cells (2×10^5 cells) were seeded into each flask and grown/incubated 24 h prior to treatment. The viable cells number for each sample was based on trypan blue exclusion, which was determined [28]. The ratio of number of viable cells/all cells results in the percentage of viable cells. Dose was considered cytotoxic when cell survival was <70%.

Cytotoxicity Assay – MTT: The different cell lines (HT-29 and Vero cell) were seeded into 96-well tissue culture plates at a concentration of 5×10^3 cells/100 µL/well and could grow overnight. The cells were subsequently treated with serial dilutions of CHE. After 48 h of incubation, the cell viability of each cell line was determined by an MTT colorimetric assay [29]. Briefly, 100 µL of 2 mg/mL MTT reagent (Sigma-Aldrich, St Louis, MO, USA) was added to each well, and the mixture was incubated for 4 h at 37°C. Then, the media was removed and 100 µL of dimethyl sulfoxide was added to each well. Finally, the OD595 of each well was measured by an ELISA reader (SpectraMAx Paradigm – VERSAmax tunable microplate reader). Cell viability was plotted as a percentage of that of the untreated control and the 50% inhibitory drug concentration (IC50) was determined from the dose–effect curve.

Comet assay

Nucleated cells from peripheral blood were obtained from 8 mL of human peripheral blood of a healthy male non-smoking individual, aged 35. Blood was transferred to tubes containing anti-coagulant and centrifuged for 10 min at 1000 × *g*. The buffy coat was collected along with red blood cells and serum. Cells were then transferred to a sterile micro-centrifuge tube and stored protected from light at room temperature until the start of micro-cultivations. The Comet Assay was performed based on the protocol [30].

Two persons analyzed each sample twice by optical microscopy (100-fold magnification). In each of the two slides, 100 comets were analyzed and classified as 0, 1, 2, 3 and 4, according to the size of their tail type [30]. The index of genetic damage at each CHE concentration was determined by summing the number of multiplications of comets with their respective class, and the percentage of damage in each treatment.

Anti-genotoxic activity using comet assay

Anti-genotoxic activity was measured with the Comet assay using human peripheral blood cells (leukocytes) [30]. Cells were pre-loaded with CHE (24 mg/mL) for 1 to 4 h, collected by centrifugation and twice washed with saline solution before being re-suspended in RPMI 1640 medium and exposed for 5 min to 4NQO (1 mM) at 37°C. Saline (0.9% NaCl) was used as negative control.

Assays with *S. cerevisiae*

To confirm the previous results of toxicity and verify that the biofertilizer had some other metabolic pathway of action, we performed the test using mutant strains of *S. cerevisiae.*

1 Strains and media: Relevant genotypes of yeast strains used in this work are given in Table 2. Media and solutions were prepared [31,32]. Complete liquid medium (YPD – 1.0% yeast extract, 2.0% polypeptone, 2.0% glucose, pH 5.3) was used for routine growth of yeast cells. For plates, the medium was solidified with 2% agar (YEL).

Yeast growth and treatments: Stationary phase (STAT) cultures were obtained by inoculation of an isolated colony in liquid YPD and incubation at 30°C for 48 h. Exponential growth (LOG) were obtained by inoculation of 5×10^5 cells/mL of an YPD culture in stationary phase into fresh 50 mL SC medium. For all the experiments, the cells in STAT or LOG phase were washed twice with saline solution (0.9% NaCl, pH 5.0) and a saline suspension of 2×10^8 cells/mL was incubated at 30°C for 48 h with 5 or 10 mg/mL of CHE. After CHE exposure cells were washed in saline and, after appropriate dilution, plated for determination of survival. Negative control used cells treated with saline (0.9% NaCl).

Statistical test

Statistical analyses were performed when differences in results

Strain	Genotype				Protein lacking			Source
BY4741	*MATα;*	*his3*	*1;*	*leu2 0;*	None	test	relevant	EUROSCARF
	lys2 0;	*ura3*	*0*		(Wildtype)			
xrs2	Like	BY4741		Except	Protein	required	for DNA	EUROSCARF
	xrs2	: *kanMX4*		repair; component of *Mre11 complex*				
mdl1	Like	BY4741	except	Mediates export of peptides generated upon proteolysis of mitochondrial proteins export of peptides generated upon proteolysis of mitochondrial proteins				EUROSCARF
	mdl1							
bpt1	Like	BY4741	except	ABC type transmembrane transporter of MRP/CFTR family; involved in the transport of heavy metal detoxification.				EUROSCARF
	yol017c	*kanMX4*		Transporter of MRP/CFTR family involved in the transport of heavy metal detoxification.				
pxa1	Like	BY4741	except	Required for import of long- chain fatty acids into peroxisomes				EUROSCARF
	pxa1	*kanMX4*						
ycf1	Like	BY4741	except	Vaculor gluthathione S-Euroscarf conjugate transporter; ABC- C transporter of the ATP-binding cassette family; required for vacuole fusion				EUROSCARF
	ycf1	: *kanMX4*						

Table 2: Yeast strains used.

were observed. Error bars represent standard deviation as calculated by the GraphPad Prism® 5 program (GraphPad Software Inc. San Diego, CA). Values represented in graphs are the average of three experiments ± SE followed by ANOVA - Tukey's analyses to compare means, were only performed in hemolytic test, larvicidal activity and comet assay (genotoxic and anti-genotoxic activity).

Results and Discussion

Our first tests evaluated the bacteriocidic, fungicidic, anti-viral and inseticidic potential of CHE considering that it is used as a biofertilizer, and therefore in direct contact with soil, which supports different types of crop [33,34].

The Minimum Inhibitory Concentration (MIC) for bacteria and yeast revealed no interference of CHE on these microorganism's growth in concentrations ranging from 0.023 to 3.0 mg/mL. Compounds present in sub fractions of CHE have been shown to have antimicrobial activity on gram-negative bacteria (*Klebsiella pneumoniae, Salmonella choleraesuis*, and *Pseudomonas aeruginosa*) at concentrations around three times higher (10 mg/mL) compared to the concentration used in our study [2]. Nevertheless, it is worth to note that higher CHE concentrations were not used because of its dark color makes visualization and test absorbance reading difficult. The maximum concentration used by us was, however, six times higher as compared to the 0.5 mg/mL recommended for MIC tests by the CLSI [16].

Antimicrobial tests were also conducted incorporating cocoa pod husk in the chemical composition of soap based on *Aloe vera*. This addition showed no effect against gram-positive and gram-negative bacteria at concentrations of 6.25, 25 and 100 mg/mL final product [35]. compounds are classified according to the exposure concentrations needed for toxicity: when values are <500 µg/mL, they exhibit strong antimicrobial activity; between 600-1500 µg/mL moderate activity; and >1,600 µg/mL they are rated as weak inhibitors or without activity [36].

The CHE bio-fertilizer also showed no antifungal activity (yeast) at <3.0 mg/mL. These data corroborate the findings of other authors, who claim that cocoa pod husk had no relevant antifungal activity on the yeast *C. albicans* as well as on filamentous fungi, i.e., *Aspergillus clavatus* and *Trichophyton spp*. [35]. Some authors report that CHE and the non-processed pod husks reduce the incidence of filamentous fungi such as *Phytophthora palmivora* and *M. perniciosa*. However, concentrations used by these authors were very high (200 mg/mL) [2,37].

The antiviral activity was tested with the EHV-1 virus and Vero cells as a host. The results showed that the value of IC50 antiviral activity was 1.29 mg/mL and CC50 was 3.19 mg/mL, thereby obtaining a SI value of 2.47. The obtained SI value of 2.47 indicates that the extract has not shown antiviral activity but the safety of the test compound since toxicity to virus was higher as compared to infected cells without treatment [38]. SI values >4 are considered positive and the higher this value, the more antiviral potential is given to the extract [21,39].

The insecticidal activity of CHE was tested against *A. aegypti* larvae at 5 different concentrations ranging from 0.75 mg/mL to 12 mg/mL. After 24 h, there was 6% death (from 136.9 coefficient of variation) for the highest concentration tested. No dead larvae were observed prior to that time and none at lower concentrations. These data show that CHE had no insecticidal activity thus can be justified as environmental-friendly. Some insects have an important function in nature, from allowing natural reforestation through pollination to composing a base of the food chain of many reptiles. Furthermore, it is known that various compounds are applied directly to the soil, either as fertilizer or for biocidal purposes, such as the CHE. However, some of them end up indiscriminately killing insects, which can result in negative environmental impacts [40].

Some fertilizers have a negative impact on the handler's health and may, during long-time exposure, cause life-threatening damage. Cellular toxicity and carcinogenicity are among the mainly listed negative impacts of such fertilizers [41]. Based on this reasoning, tests were conducted to evaluate the potential toxicity, cytotoxicity and genotoxicity of the cocoa pod husk-extracted biofertilizer CHE.

Toxicity (*A. salina*) and cytotoxicity (HT-29 and Vero cell) assays have shown that even in high concentration (6.0 mg/mL), CHE biofertilizer was not toxic. These tests establish a correlation between

the degree of toxicity and the median lethal dose (LD50) of the compound. When values >1,000 µg/mL are needed to obtain toxicity and death is not above 50%, the administered substance is considered non-toxic [42].

The Hemolysis Test (Figure 1) was based on *in vitro* destructive interaction of CHE with the erythrocyte membrane. Therefore, washed erythrocyte samples were exposed to 3 concentrations of CHE (1.5, 3 and 6 mg/mL). The results showed that CHE had dose dependent low hemolytic activity when compared to control.

Hemolytic activity against human erythrocytes is determined in a dose-dependent manner to obtain the concentration at which it causes 50% hemolyses as compared to Triton X-100 treatment (HC50). Based on 11% homolyses after 6 mg/mL CHE exposure with can calculate by extrapolation a HC50 for CHE of 27 mg/mL. These results demonstrate the low hemolytic activity of CHE and its concomitant safety.

Genotoxic and protective assessment tests were carried out using the Comet Assay. Results indicated that potential damage production in leukocyte DNA was relatively low as compared as positive control (Figure 2). There was significant difference ($p<0.05$) between the lowest and highest applied CHE concentration, which, however remained small as compared as to 4NQO. We conclude that regardless of the concentration used, the genotoxicity of the compound was low (Figure 2) which agrees with results found for CHE-exposed V79 cells [2]. The compound also showed no evidence of protective activity against the 4NQO (1 mM) associated with 24 mg of CHE (Figure 3).

After observing that even high doses CHE showed only low toxic activity in any of the tested cells (Figure 2), we decided to check possible effects of CHE action when applied in high concentrations. To this end we determined the sensitivity to CHE (no growth) of haploid mutant strains with defect in defined physiological pathways. After exposure of the different strains of *S. cerevisiea* to CHE concentrations of 5 (not shown) and 10 mg/mL we could not observe any change in survival (growth) amongst the different yeast strains. As all mutant strains had the same WT-like response to CHE we can assume that it most probably does not act on DNA, putative ABC transport, mitochondrial activity, and peroxidase and cell vacuole (Figure 4).

Even at CHE concentrations 10 times higher than recommended (1 mg/mL), yeast DNA repair mutant *xrs2* showed wild-type like survival. Furthermore, the biofertilizer revealed very low or no toxicity when applied to yeast mutant strains with deficiencies in various metabolic pathways (Figure 4). We may thus assume that the CHE biofertilizer does not interact with DNA, putative ABC transporter, mitochondria, peroxidase and cell vacuole functions. Haploid yeast cells harbouring *fet3* and *aft1* mutant alleles showed the same survival (85%) to 10 mg/mL CHE as the isogenic WT, while *fet4* and *zap1* mutants had wild-type-like survival. These mutations are associated with the activity of ferric and cupric reductase [2].

Conclusion

Several scientific publications report that cocoa pod husks can be used as biofertilizer, as it contains vital compounds and stimulates growth of *Theobroma cocoa* and of other plants.

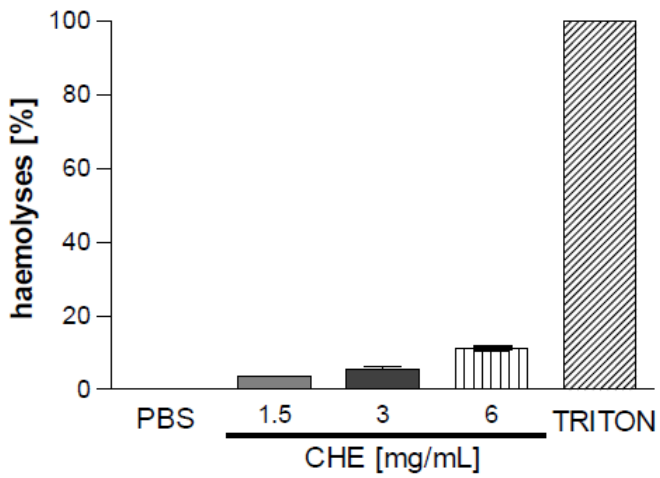

Figure 1: Haemolytic activity using human erythrocytes: CHE-treated cells (concentrations of 1.5 to 6 mg/mL,1 h, 37°C), PBS (negative control - 1%, 1.5 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 135 mM NaCl, pH-7.4, 1 h, 37°C), TRITON X-100 (positive control, 1% in PBS, 1 h, 37°C). Error bars represent mean ± SE of 3 independent experiments. $p<0.05$.

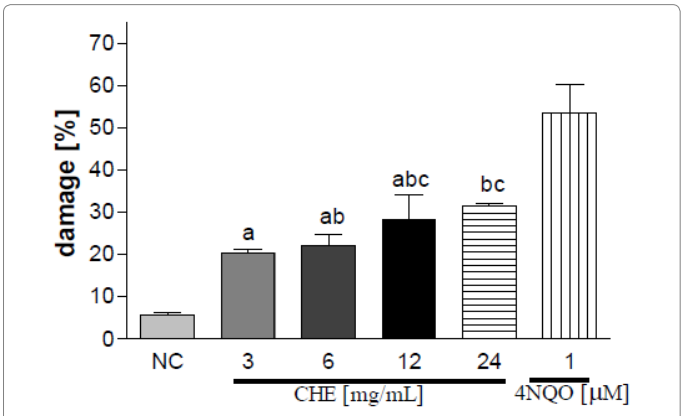

Figure 2: Genotoxic activity measured by Comet Assay of human leukocytes: CHE-treated cells (final concentration of 3 to 24 mg/mL, 1 h, 37°C), H_2O (negative control and sample diluent, 1 h, 37°C) or 4NQO (positive control, 1 µM, 1 h, 37°C). Error bars represent mean ± SE of 3 independent experiments. *p<0.05=between a and c; **p<0.05= between NC and a, b; ***p<0.05=between NC and c and 4NQO and all the others.

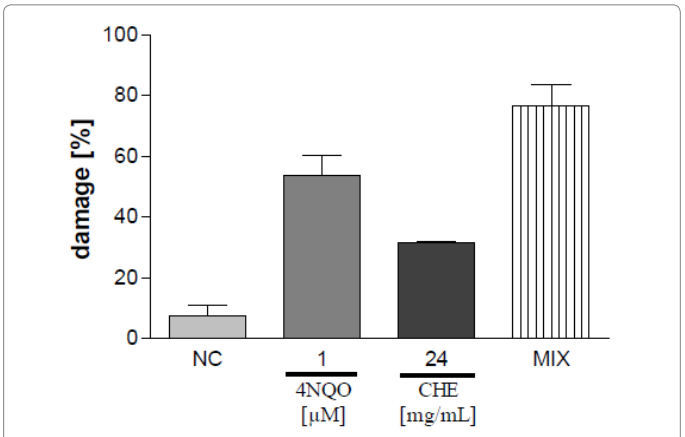

Figure 3: Anti-genotoxic activity measured by the percentage of DNA damage, as detected by the Comet Assay. Human leukocytes were preloaded with 24 mg/mL CHE for 4 h then incubated for 5 minutes in different concentrations of 1 µM 4NQO at 37°C. Data represent 3 independent experiments. ***p<0.05. NC=Negative Control (water). Mix is 1 µM 4NQO plus 24 mg/mL CHE.

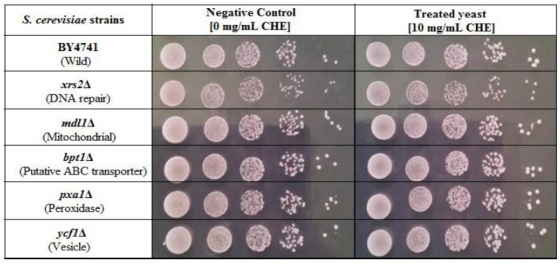

Figure 4: Effect of CHE on *S. cerevisiae* survival: CHE-treated saline solution-suspended yeast (concentrations of 0 and 10 mg/mL, 24 h, 30°C). Plated on YEL in serial ten-fold dilution.

In a battery of antimicrobial, toxic and genotoxic tests using pro- and eukaryotic microorganisms, insect larvae and human erythrocytes and leucocytes we show here that the application of liquid extract of fermented macerated pod husk may not only have no negative environmental impact but also may be safe to handle by farm workers applying this biofertilizer.

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