

Evaluation of Cocoa Bean Shell Antimicrobial Activity: a Tentative Assay Using a Metabolomic Approach for Active Compound Identification

(Dedicated to Professor Arnold Vlietinck on the occasion of his 80th birthday)

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Abstract

Cocoa bean shell (CBS) is one of the main by-products of chocolate manufacturing and possesses several compounds with biofunctionalities. It can function as an antibacterial agent, and its action is mostly reported against *Streptococcus mutans*. However, only a few studies have investigated the CBS compounds responsible for this activity. This study aimed to evaluate several extracts of CBSs from different geographical origins and cocoa varieties and estimate their antimicrobial properties against different fungal and bacterial strains by determining their minimal inhibitory concentration (MIC). The results demonstrated antimicrobial activity of CBS against one of the tested strains, *S. mutans*. CBS extracts were further analysed via liquid chromatography–high-resolution mass spectrometry (LC-HRMS) for untargeted metabolomic analysis. LC-HRMS data were analysed (pre-processing and statistical analyses) using the Workflow4Metabolomics platform. The latter enabled us to identify possible compounds responsible for the detected antimicrobial activity by comparing the more and less active extracts. Active extracts were not the most abundant in polyphenols but contained higher concentrations of two metabolites. After tentative annotation of these metabolites, one of them was identified and confirmed to be 7-methylxanthine. When tested alone, 7-methylxanthine did not display antibacterial activity. However, a possible cocktail effect due to the synergistic activity of this molecule along with other compounds in the CBS extracts cannot be neglected. In conclusion, CBS could be a functional ingredient with benefits for human health as it exhibited antibacterial activity against *S. mutans*. However, the antimicrobial mechanisms still need to be confirmed.

Keywords (4-6)

Cocoa bean shell, *Theobroma cacao*, Malvaceae, antimicrobial activity, *Streptococcus mutans*, metabolomics.

Abbreviations

AMX, amoxicillin; BHI, brain heart infusion; CBS, cocoa bean shell; FLC, fluconazole; GTF, glucosyltransferase; LC-HRMS, liquid chromatography–high-resolution mass spectrometry; MIC, minimal inhibitory concentration; NYS, nystamine; PCA, principal component analysis; PEN G, penicillin G; PEN V, penicillin V; PLS-DA, partial least squares discriminant analysis; TS, tryptic soy; W4M, Workflow4Metabolomics; YPD, yeast extract-peptone-dextrose.

Introduction

Plants have been used for their healing potential since ancient times. They have been used in folk medicine even before humans were aware of the existence of microbes and their disease-causing potential. Today, it is known that this antimicrobial potential is derived from secondary metabolites contained in the plants [1, 2]. Recently, antibacterial resistance has become a major problem leading to the focus of research on new molecules with antimicrobial potential [3]. To this end, compounds from plant materials have attracted the attention of most researchers in the last several years, and novel vegetal molecules such as polyphenols or essential oils with strong antimicrobial potential have been identified [4, 5]. Special attention has been paid to different plant-based extracts. The complexity of these mixtures of molecules and their ability to act synergistically provides them with antibacterial properties that are, in many cases, close to those of the active isolated molecules [6]. This, together with the increasing issue of food spoilage in modern societies, increases the importance of the study and revalorisation of vegetal by-products as high-value-added sources of bioactive compounds [6, 7].

In the cocoa industry, one of the main by-products of the chocolate manufacturing process is the cocoa bean shell (CBS), which represents approximately 10%–17% of the total cocoa bean weight and is normally discarded after roasting and husking of the beans [8]. CBS has been reported to be rich in bioactive compounds such as polyphenols and methylxanthines. Hence, it has been largely proposed as a nutraceutical for the development of functional foods and beverages [9-11]. Moreover, the possibility of pharmacological application of CBS has been explored. Several biofunctionalities, such as antidiabetic, anticarcinogenic, antiviral, anti-inflammatory, and most importantly, antibacterial properties, have been demonstrated for this by-product. Indeed, the antimicrobial activity of CBS extracts against different pathogens, mostly oral pathogens, has been reported recently [12-14]. However, the mechanism of action and the molecular components in the CBS extracts responsible for these activities remain unclear. To this day, polyphenols have been proposed to be the compounds responsible for this activity [4, 15].

As CBS has already been proposed to be a low-cost functional ingredient, this study aimed to test different unrelated CBS sample extracts with known polyphenolic contents against various food-related bacterial and fungal strains. The microdilution method was employed to determine the minimum inhibitory concentration (MIC), and an untargeted metabolomic approach was used to identify the possible active molecules in the CBS extracts showing MICs lower than a pharmacologically established interesting threshold (≤ 500 $\mu\text{g/mL}$) [1, 15]. For the first time, different types of CBS sample extracts were

tested for their antimicrobial activity, and their differential compositions were analysed by metabolomics. This approach allowed us to consider the chemical variability presented by the CBS samples due to different biotic and abiotic factors such as plant variety or geographical origin.

Results and Discussion

Twelve extracts of CBS yielded from different cocoa beans of diverse origins and varieties and with variable and known polyphenolic content (Table 1) were tested against different bacterial and fungal strains (Table 2). The MICs of the different antibiotics used as positive controls against the eight fungal and bacterial strains are shown in Table 1S (Supporting Information). CBS extracts were tested at concentrations between 3.91 and 500 µg/mL; the latter (500 µg/mL) was the threshold concentration for considering a plant extract as 'pharmacologically active' [1, 15]. Consequently, CBS extract concentrations higher than 500 µg/mL were not interesting from a pharmacological point of view and were not considered.

For most strains tested in this study, no inhibition was found below 500 µg/mL (MSSA, MRSA, resistant and sensitive *Escherichia coli*, *Pseudomonas aeruginosa*, *Saccharomyces cerevisiae*, and *Candida albicans*). Some researchers have previously reported activity from few cocoa-related products such as cocoa powder, cocoa pods, or even CBS extracts against these strains. However, in most cases, this activity was found at higher concentrations than those considered in this study. Pectin extracts from cocoa pods showed MICs of 1.0, 1.0, and 2.0 mg/mL against *E. coli*, *S. aureus*, and *P. aeruginosa*, respectively [16], while other researchers found a MIC of 5.0 mg/mL of fermented cocoa pod extracts against *P. aeruginosa* [17]. However, Todorovic et al. [18] found a MIC of 5 mg/mL against *C. albicans* and MICs between 12 and 20 mg/mL against *E. coli*, *S. aureus*, and *P. aeruginosa* for cocoa powder extracts. These concentrations decreased significantly (up to 8.5–11.5 mg/mL) when alkalisated cocoa powder samples were used. Therefore, they associated this activity with some electronegative flavour-related compounds such as aldehydes and ketones whose concentrations increased after alkylation. Using CBS acetone extracts, Nsor-Atindana et al. [12] found MICs of 0.468 and 0.937 mg/mL against *E. coli* and *S. aureus*, respectively. However, CBS ethanol extracts similar to those prepared in our studies showed MICs of 1.875 mg/mL for both bacteria. Nonetheless, it was stated that those activities were not

correlated to the phenolic levels found in the CBS extracts. In all cases, active concentrations were higher than the threshold of interest (0.5 mg/mL) considered in this study.

In *S. cerevisiae*, inhibition of this desirable microorganism was not detected at the tested concentrations. Nonetheless, an increased growth was observed with increasing CBS concentrations (Figure 1). Thus, CBS extracts were not harmful to the yeast, but rather the opposite was true. These results were consistent with the presence of *S. cerevisiae* in the naturally occurring fermentation of cocoa beans [19]. Indeed, during the CBS extraction process with an ethanol/water mixture, the sugars contained in CBS could also have been extracted. These sugars may serve as nutrients for the yeast. Moreover, the confirmation that this yeast was not inhibited by the CBS components was important from a food technology point of view since the valorisation of this by-product as a cocoa powder substitute in baked goods employing yeast has already been reported [20, 21].

Regarding the gram-positive bacteria *Streptococcus mutans*, 11 out of the 12 different CBS sample extracts displayed MICs of 500 µg/mL or less (Table 3). Therefore, they were considered of pharmacological interest and further studied for the tentative identification of their active components. *Streptococcus mutans* is a well-known bacterial strain associated with dental caries [22]. Several studies have already reported the anticariogenic effects of CBS at concentrations of 1 mg/mL [13]. Some researchers have even proposed its use in oral care as a mouthwash for caries prevention with an efficacy significantly similar to that of the chlorhexidine mouthwashes [8]. The MICs of extracts from fermented and unfermented cocoa beans and cocoa liquor against *S. mutans* have already been reported (8, 4, and 8 mg/mL, respectively) [23]. However, although the cariostatic effects of CBS have been reported, there is no specific information in literature about its *in vitro* MIC. To our knowledge, this is the first study in which these particular values for this cocoa by-product have been reported.

Amongst all the microbial strains tested, considerable activity was found only against the cariogenic bacterial strain *S. mutans*. Indeed, all the tested CBS samples, except for extract number 5, had MICs ≤500 µg/mL (Table 3). Notably, three extracts were more effective than the others were. Extracts 9, 11, and 12 showed MICs of 125, 62.5, and 125 µg/mL, respectively (Figure 2). Thus, these three extracts were compared to evaluate the differences in their composition and that of the remaining samples for identification of the active compounds. To achieve this, all CBS extracts were analysed through LC-HRMS, and the obtained data were treated in the Workflow4Metabolomics (W4M) platform using an

untargeted metabolomic approach. Extracts numbered 9, 11, and 12 were considered 'active' and the remaining samples were considered 'not active' while performing different statistical analyses. Two types of multivariate analyses were applied to the data: principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) (Figure 3). Supplementary information on these plots is provided in Figure 1S (Supporting Information). PCA, which is an unsupervised analysis, did not allow for sample separation depending on the different activities against *S. mutans*. The PLS-DA, which is a supervised analysis that builds the model with the help of the provided data, allowed instead for a clear separation of the two groups of samples. Besides, univariate analyses were performed using *t*-test as test method with a false discovery rate (FDR) < 0.05 to understand how the concentration of some molecules of interest varied depending on the different CBS samples.

Independent studies describing the antibacterial activity of CBS against different strains have attributed it to the polyphenolic content [4, 7, 24]. It has been claimed that some polyphenols, such as those found in tea, can inhibit acid production by *S. mutans*, which is the main cariogenic cause of this strain when adhered to dental surfaces [25]. Additionally, several authors have reported the possible antibacterial activity of catechin and epicatechin, contained in cocoa and CBS, by interaction with the bacterial cell membranes. The negative charge makes them bind strongly to the positively charged lipid bilayer of the gram-positive bacteria [18]. Moreover, these molecules are believed to undergo oxidative polymerisation and form molecules that could further polymerise with proteins and sugars. This produces high molecular weight compounds that have been reported to inhibit the glucosyltransferases (GTFs) of *S. mutans* [14, 26, 27]. Indeed, GTFs are the most significant virulence factors of *S. mutans* and are highly involved in its pathogenicity. They are responsible for the sucrose-dependent synthesis of water-insoluble glucans that ultimately lead to bacterial adhesion on the tooth surface. They form a cariogenic dental plaque biofilm and further cause the demineralisation of the dental surface by the adhered bacteria metabolic acid production [22, 28, 29]. However, in this study, we found that the antibacterial activity of CBS against *S. mutans* was not directly related to the total content of polyphenols. Indeed, CBS extract number 11, which showed the highest activity against *S. mutans*, was one of the samples chosen for the comparative analysis because it had the lowest amount of polyphenols amongst the total samples (Table 1). Sample 5 chosen under the same criterion showed instead the highest MIC (lowest activity). Surprisingly, while performing univariate tests in our study, both catechin and epicatechin concentrations were found to be significantly lower in the most active extracts (Figure 4a and 4b). Further, standards

were tested against *S. mutans* using concentrations (31.25–4000 µg/mL) greater than those of pharmacological interest to assess their possible contribution to the antibacterial activity. Catechin and epicatechin showed MICs against *S. mutans* at concentrations of 4 and >4 mg/mL, respectively (Table 3). Thus, they were both not active enough to be considered as the compounds responsible for the antibacterial activity of CBS.

Furthermore, Ferrazzano et al. [27] reported that caffeine could interfere with the adsorption of *S. mutans*. However, this factor was also excluded in this study because the differences in the main methylxanthines (theobromine and caffeine) contained in CBS were not significant amongst the different extracts (FDR > 0.05; Figures 4c and 4d, respectively). Additionally, caffeine and theobromine standards were tested for their MICs, and the values were found to be 4 and >4 mg/mL, respectively (Table 3). Therefore, they were not considered pharmacologically active factors against *S. mutans*.

As indicated before, a separation by PLS-DA was achieved with the employed metabolomic approach. This separation proved to be driven by some principal features. Herein, two specific molecules presented the highest variable importance in the projection (VIP) coefficients of the model with all components. These were then considered responsible for the separation of the most active CBS extracts from the less active ones: $m/z = 167.0557$, $t = 203$ s and $m/z = 369.2042$, $t = 1344$ s (Figures 4e and 4f, respectively). These molecules were indeed present in significantly higher concentrations in the CBS extracts that were considered 'active' against the *S. mutans* strain.

The first feature (M167T203) was further confirmed to correspond to 7-methylxanthine (Figure 4e), a theobromine and caffeine precursor that was identified in CBS for the first time. Unfortunately, when testing the 7-methylxanthine alone against *S. mutans*, no direct inhibition was observed below 4 mg/mL (Table 3). However, a possible synergistic effect of this compound along with other bioactive compounds in CBS (mainly polyphenols) was not disregarded because of the higher presence of 7-methylxanthine within the active CBS extracts. Unfortunately, we were unable to provide a specific identification for the second feature (M369T1344, Figure 4f). Nevertheless, four different suppositions for the identity of the molecule have been done based on the data found in the European MassBank database (<https://massbank.eu/MassBank>) and further investigations should be carried out for confirmation of feature identity. According to its mass, this possibly active molecule could be curcumin (C₂₁H₂₀O₆), a polyphenol that has already been reported to possess short-term and long-term antibacterial effects against *S. mutans* and display no visible bacterial growth at an MIC of 125 µM (approximately 46 µg/mL) [30].

However, curcumin has never been reported in cocoa or cocoa-similar products. Another possibility is feruloylquinic acid (C₁₇H₂₀O₉). This molecule is mainly present in coffee but, similar to curcumin, has never been detected in cocoa products. However, it has been previously reported that ferulic acid does not show any activity against *S. mutans* and that, therefore, feruloylquinic acid does not probably exert such activity neither [31]. Nevertheless, the quinic fraction could make the activity of feruloylquinic acid and ferulic acid different from each other and this fact should be confirmed. The third hypothesis points to a prenylated flavone (C₂₁H₂₀O₆). This could be a very reasonable option since prenylated flavonoids have been reported to strongly inhibit bacterial and fungal activities. Prenylation increases the lipophilicity of flavonoids and consequently increases their affinity for biological membranes. This facilitates their attachment and improves interactions with target proteins [32]. Additionally, it has been proposed that flavones could form a complex with bacterial cell wall components, which would inhibit further adhesion and growth [33]. The fourth option for the molecule conducting the PLS-DA separation according to the feature mass would be lignoceric acid (C₂₄H₄₈O₂). This unsaturated fatty acid has already been detected in CBS [34]. Similar to the prenylated flavone, this is a reasonable hypothesis because many researchers have reported the ability of several fatty acids to inhibit bacterial growth and specifically *S. mutans* [35]. Antibacterial properties of free fatty acids are well recognised. They are manifested through different pathways such as the disruption of the electron transport chain and oxidative phosphorylation in the cell membrane, inhibition of certain enzymes, inhibition of nutrient uptake, formation of peroxidation or auto-oxidation products, or direct or induced lysis of bacterial cells [36].

In summary, CBS extracts did not show a pharmacologically interesting activity (MIC ≤ 500 µg/mL) against most bacterial and fungal strains tested in this study (MSSA, MRSA, resistant and sensitive *E. coli*, *P.aeruginosa*, *C. albicans*, and *S. cerevisiae*). However, interestingly, when tested against *S. cerevisiae*, higher CBS extract concentrations promoted fungal growth. This could be important in the application of this by-product in food product development wherein yeast action is desired. *Streptococcus mutans* was the only strain against which CBS extracts showed inhibition at pharmacologically interesting concentrations. However, significant differences in the MIC values were observed amongst the extracts tested. In contrast to what was expected, the most active extracts did not contain the highest amounts of polyphenols. Therefore, this factor was not directly related to the antibacterial activity of the CBS extracts. Untargeted metabolomic analysis was performed based on the data obtained by the LC-HRMS analyses of all the extracts and served to elucidate the possible active molecules responsible for

the separation of the most active CBS extracts against the cariogenic bacterial strain *S. mutans* from the less active CBS extracts depending on the differences in MIC values. Two molecules were found to be responsible for this separation and were significantly present in the active CBS extracts. The first of these molecules was identified as 7-methylxanthine, a precursor of theobromine and caffeine. Although no direct antibacterial activity was found when testing this molecule alone against *S. mutans*, a possible synergistic effect of 7-methylxanthine with other CBS extract components and polyphenols can be hypothesised. The structure of the second and putative active molecule driving the separation was not completely elucidated. However, according to its mass, four different hypotheses were proposed: curcumin, feruloylquinic acid, prenylated flavone, and lignoceric acid. The last two hypotheses were more plausible according to the known composition of CBS. Nevertheless, a complete elucidation of the antimicrobial mechanisms, as well as the study of the antimicrobial activity of CBS extracts on the biofilm formed by *S. mutans* remain as future perspectives for the present study.

Materials and Methods

Chemicals, media, and microorganisms

Dimethyl sulfoxide (DMSO; 99.9%), bacterial and yeast growth media (tryptic soy, brain heart infusion, and yeast extract-peptone-dextrose agars and broths), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), (+)-catechin hydrate (>98%), epicatechin (>98%), caffeine (≥99%), 7-methylxanthine (≥98%), MS-quality formic acid, and MS-quality methanol were obtained from Sigma-Aldrich.

The 0.85% NaCl physiological solution (2 mL) was obtained from BioMérieux.

Amoxicillin, penicillin V, penicillin G, fluconazole, nystamine and theobromine (99%) were provided by Alfa Aesar.

Ethanol (99%) was purchased from VWR chemicals.

Ultrapure water was prepared in a Simplicity UV water purification system (Millipore).

Bacterial and fungal strains were purchased from the Belgian Coordinated Collection of Microorganisms (BCCM). Details concerning the employed strains, the culture media used, and antibiotics used as positive controls are shown in Table 2.

Plant material

Twelve different CBS samples procured from fermented, dried, and roasted cocoa beans of different geographical origins within the American and African continents and different cocoa varieties (Forastero or Trinitario) (Table 1), purchased from several local cocoa companies within the Piedmont region (Italy), were selected from the 44-sample set employed by Barbosa-Pereira et al. [37, 38]. The sample selection was based on the polyphenolic content previously determined by RP-HPLC-PDA analysis when following the analysis methodology used by [10]. The total quantities obtained for the sum of all the detected and quantified polyphenols in each CBS sample as well as their origins and varieties are shown in Table 1. Ten samples (samples 1–4, 6–10, and 12) were chosen as they contained the highest determined polyphenol contents in the sample set, while the other two (numbers 5 and 11) were chosen for possessing the lowest ones.

CBS extract preparation

Extractions were performed by adding CBS powder (0.5 g) to 10 mL of an ethanol-water mixture (50:50, v/v). The extraction was performed at room temperature (25 °C) with constant rotatory oscillation for 2h in an SM 25B-SWIP laboratory shaker (Edmund Bühler). Samples were then centrifuged at 4200×g for 10 min in a 4226 ROTOFIX Centrifuge (Hettich), and the supernatants were collected and filtered through a 0.22-µm PTFE filter. The ethanol from the extracts was evaporated for 10 min in a rotavapor (Pleuger Büchi), and water was eliminated using an Epsilon 1–6 freeze dryer (Christ).

MIC assays

MIC studies were performed following the M07-A10 standardised protocol described in the Clinical and Laboratory Standards Institute guidelines [39] by employing the broth microdilution technique in 96-well plates, adapted as described previously [5]. Briefly, CBS extract stock solutions of 1 mg/mL were prepared in DMSO (3%) and the corresponding broth medium. The stock solutions were serially diluted two-fold to obtain a final concentration range of 500–3.91 µg/mL. Antibiotic stock solutions were used as positive controls and were prepared similarly with an initial concentration of 128 µg/mL, and a final concentration gradient ranging between 64 µg/mL and 1 µg/mL was obtained. For the compound standards (catechin, epicatechin, theobromine, caffeine, and 7-methylxanthine), an initial stock concentration of 8 mg/mL was used, and the concentration gradient was 4000–31.25 µg/mL. A bacterial

suspension of 24-h-old colonies prepared in 0.85% NaCl physiological solution with 0.5 McFarland-equivalent turbidity was placed in each well with a final concentration of approximately 5×10^4 CFU/well and then incubated overnight at 37°C. Additionally, growth and non-growth controls were prepared. After 24h of incubation, the MIC was observed with the naked eye and 30 μ L of MTT (0.8 mg/mL) was used for staining when needed. All experiments were performed in triplicates. MIC values were taken as the lowest extract concentrations that produced no visible bacterial growth after incubation.

LC-HRMS and metabolomic study

A methanolic resuspension of the sample extracts (1mg/mL) was used for the LC-HRMS analyses. Analyses for the untargeted metabolomic study were performed using a 1200 series Rapid Resolution LC system (RRLC, Agilent Technologies) coupled to a 6520 series electrospray ionization (ESI) source with a quadrupole time-of-flight (Q-TOF) mass spectrometer from Agilent Technologies. The separation was performed using an InfinityLab Poroshell 120 EC-C18 column (2.1×100 mm, particle size 2.7 μ m, Agilent Technologies) with a guard column (2.1×5 mm; Agilent Technologies). Water acidified with 0.1% formic acid (solvent A) and methanol acidified with 0.1% formic acid (solvent B) served as the mobile phases. The separation method and ESI-QTOF settings for data acquisition (positive mode) were performed as described previously [40]. Data acquisition and analysis were carried out using the B.04 SP3 version of the MassHunter Acquisition® software for QTOF and the B.08 version of MassHunter Qualitative Analysis® software (Agilent Technologies), respectively. Samples were randomly analysed in triplicates in a single batch. The same quality control (QC = pool) sample for all samples (mix of all samples) was injected throughout the run after every 10 samples for control; blanks (methanol) were also injected throughout the run.

The obtained Agilent .d format data were converted into .mzXML format using the ProteoWizard MSConvert software (version 3.03.9393, 64-bit) with the Peak Picking filter option. Pre-processing (peak detection, identification, grouping and smoothing, retention time correction, filtration, and integration) and quality control (metabolite correlation analysis) were conducted using the freely available Galaxy workflow4metabolomics W4M platform (<http://workflow4metabolomics.org>, [41]). The parameters employed during the workflow were identical to those used in a previous study [42].

The W4M platform was used for statistical analysis. The pre-processed data were subjected to multivariate modelling (PCA and PLS-DA) and univariate testing (*t*-test, FDR < 0.05). According to the

procedure reported by Souard et al. [42], a mean centering and unit-variance scaling was selected for multivariate testing, 20 permutations were used in the case of PLS-DA and the selected number of cross-validation segments was 7. The default algorithm was used in the W4M multivariate testing, which means 'svd' for PCA and 'nipals' for PLS. Feature selection was performed by using the Biosigner algorithm [43], which is a molecule signature discovery tool implemented in the W4M platform.

Supporting Information

The following material is provided as Supporting Information: **Table 1S.** MIC values ($\mu\text{g/mL}$) for all the tested microorganisms against the different antibiotics used as positive controls ($n = 6$). **Figure 1S:** Supplementary information on the score plots for multivariate modelling using PCA or PLS-DA.

Acknowledgements

O. Rojo-Poveda gratefully acknowledges the University of Turin for the award of a PhD student fellowship (S102.190016) in co-supervision with the Université libre de Bruxelles, and the ULB Research Department for the mobility and PhD-end grants.

L. Barbosa-Pereira is grateful to the European Union's Seventh Framework Programme for the Marie Skłodowska-Curie grant and to the Spanish Ministry of Science, Innovation and Universities for her “Juan de la Cierva—Incorporación” Grant (Agreement No. IJCI-2017-31665).

The LC-HRMS equipment has been supported by the Belgian National Fund for Scientific Research (grants FRS, N° 3.4553.08 and T.0136.13 PDR) and Université libre de Bruxelles (FER-2007). The authors are grateful to the Microbiology, Bioorganic and Macromolecular Chemistry Unit of the ULB for allowing microbiological assay performance in their biosafety level facilities. The authors thank M. Faes and O. Vaillant for their assistance.

The authors are grateful to Silvio Bessone S.r.l., ICAM S.p.A., Ferrero International S.A., Guido Gobino S.r.l., and Pastiglie Leone S.r.l. for supplying the cocoa bean samples.

Conflict of Interest

The authors declare no conflict of interest.

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Figure 1. MIC assay of *S. cerevisiae* showing more fungal growth (identified as turbidity, highlighted by the red square) when more CBS extract is present (gradient from top to bottom). Dark areas can be distinguished in less concentrated wells showing less yeast growth.

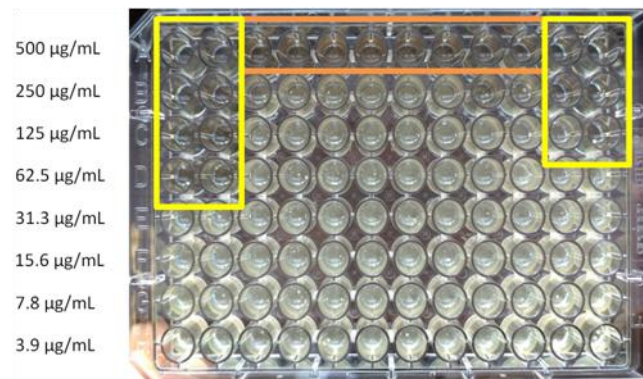


Figure 2. MIC assay conducted with *S. mutans*. Clear wells, marked in yellow and showing no turbidity, indicate the MICs obtained for extracts 11 (at left) and 12 (at right) that were considered ‘active’ during the metabolomic analysis (62.5 and 125 µg/mL, respectively). The upper clear wells, highlighted in orange, display MICs of 500 µg/mL for the other CBS extracts considered as ‘not active’ in the metabolomic analysis.

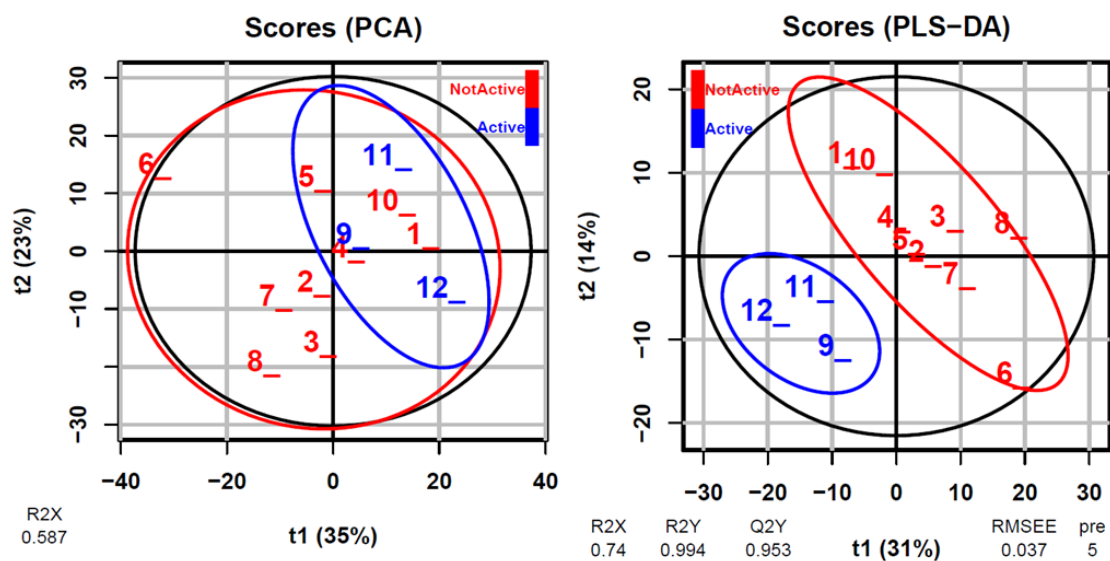


Figure 3. Score plots for the multivariate modelling of variations using principal component analysis (PCA) or partial least squares discriminant analysis (PLS-DA) of all CBS extracts ($n = 3$) according to their activity level against *S. mutans*. Extracts number 9, 11, and 12 were considered ‘active’ while the remaining extracts were considered ‘not active’ during both multivariate analyses. For each plot, the percentages of total variation explained by components 1 and 2 (t1 and t2, respectively) are indicated in parentheses. Black ellipses include 95% of the multivariate normal distribution for all samples (subgrouped in coloured ellipses according to the antibacterial activity level against *S. mutans*). Supplementary information for these plots is given in Figure 1S, Supporting Information.

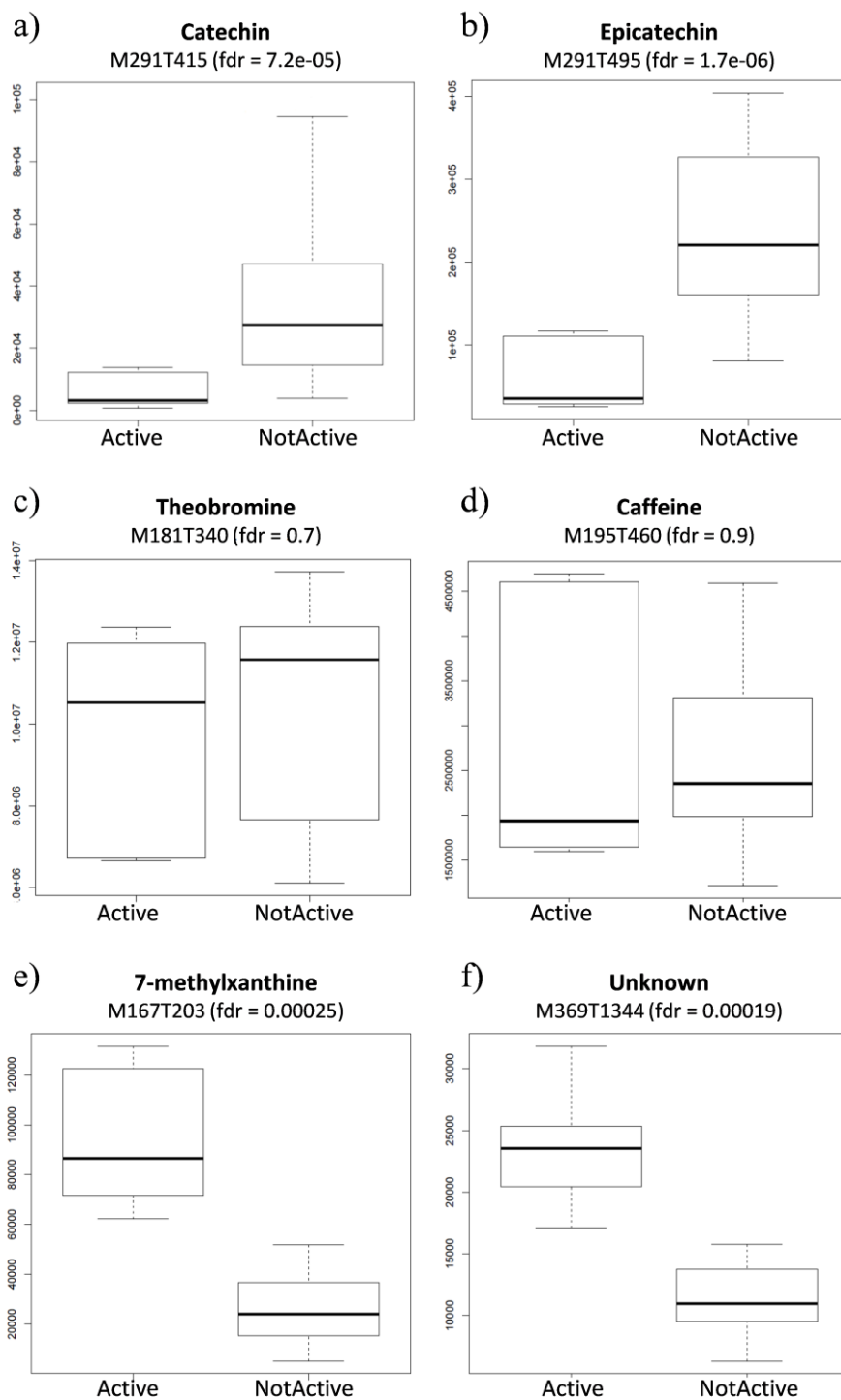


Figure 4. Univariate analysis boxplots of the log₁₀-intensities of (a) catechin ($m/z = 291.0856$, $t = 415$ s), (b) epicatechin ($m/z = 291.0859$, $t = 495$ s), (c) theobromine ($m/z = 181.0713$, $t = 340$ s), (d) caffeine ($m/z = 195.0669$, $t = 460$ s), (e) 7-methylxanthine ($m/z = 167.0557$, $t = 203$ s), and (f) the putative active molecule ($m/z = 369.2042$, $t = 1344$ s) for the two groups of the CBS extracts considered either active (9, 11, and 12) or not active (1–8 and 10) (t -test, significant when $fdr < 0.05$).