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Evaluating the Antifungal Efficacy and Bioactive Metabolites of a Traditional Polyherbal Aqueous Formulation BioCC+ against Fusarium graminearum and Fusarium verticillioides

--Manuscript Draft--

Evaluating the Antifungal Efficacy and Bioactive Metabolites of a Traditional Polyherbal Aqueous Formulation BioCC+ against *Fusarium graminearum* and *Fusarium verticillioides*

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Abstract

Fungal contamination of cereal crops threatens global food security by compromising grain quality and introducing harmful mycotoxins that endanger human and animal health. In West Africa, the long-standing ancestral use of native plant extracts to manage fungal contamination provides sustainable and locally adaptable solutions. Building on this knowledge, BioCC+, a polyherbal aqueous formulation containing *Azadirachta indica*, *Cymbopogon citratus*, *Allium sativum*, and *Capsicum frutescens*, was developed to control maize contamination during storage in Côte d'Ivoire. This study evaluated the antifungal activity of BioCC+ against *Fusarium graminearum* and *F. verticillioides* followed by bioactivity-guided untargeted metabolomics to explain observed effects. Crude BioCC+ extracts inhibited *F. graminearum* at 12.5 mg/mL and *F. verticillioides* at 25.0 mg/mL, with the dichloromethane extract showing greater antifungal activity than methanol or water extracts. Based on the observed growth inhibition the dichloromethane extract (DCMe) was selected for fractionation using silica gel column chromatography with the following mixtures of hexane:ethyl acetate as the mobile phase: 100:0 for F1, 75:25 for F2, 50:50 for F3, 25:75 for F4, and 0:100 for F5, as well as 100% methanol for F6. Fractionation of DCMe revealed Fractions 3, 4, and 6 as the most active, with Fraction 4 showing the strongest inhibition (MIC = 0.4 mg/mL) against *F. graminearum*. Integration of antifungal and chemometric data through multivariate analysis identified 26 potential antifungal compounds, including 13 uncharacterized metabolites. These results demonstrate the value of integrating bioactivity assays with metabolomics to validate ancestral knowledge and develop community-driven practices for mitigating fungal contamination in staple crops.

Keywords: Mycotoxins, Biofungicide, Fungal inhibition, *Fusarium* spp., Metabolomics, Biochemometrics

Data Summary

All data generated or analyzed in this study are either presented in the manuscript or publicly accessible in the ScholarSphere data repository (1).

Impact Statement

This study integrates antifungal bioassays with untargeted metabolomics to identify 26 bioactive metabolites in a prototype biofungicide derived from ancestral plant mixtures from Côte d'Ivoire. By validating traditional practices with modern biochemometrics, our findings support the development of sustainable, locally adapted antifungal solutions for reducing mycotoxin contamination and improving food security.

Introduction

Rice, maize, millet, sorghum, and wheat are staple cereal crops grown in diverse agroecological zones and consumed by people with varying preferences and socio-economic backgrounds in sub-Saharan Africa (2). The agricultural sector of Côte d'Ivoire contributes 20-25% to the country's Gross Domestic Product and employs about 48% of the population (3). Côte d'Ivoire's economy is heavily dependent on the export of cash crops such as cocoa, coffee, and cashew nuts, while cereal crops play a crucial role in food security and supports the local economy (3). However, cereal crops are susceptible to post-harvest contamination by filamentous fungi that produce mycotoxins, which pose a threat to human and animal health. For example, fusarium toxins produced by *Fusarium graminearum*, *F. verticillioides*, and *F. culmorum*, and aflatoxins produced by *Aspergillus flavus* and *A. parasiticus* are regularly implicated in cereal contamination, both in the field and during storage (4,5). In addition to adverse effects on human and animal health, mycotoxin contamination of cereal crops results in considerable economic losses and a reduction in the income of farmers and growers (6). To

reduce the burden of post-harvest fungal contamination in modern agriculture, countries with higher socio-economic status rely on the use of synthetic chemicals as fungicides. However, misuse and overuse of fungicides can be expensive, lead to fungal resistance, and further negative effects on both human and environmental health (7). Therefore, innovative, eco-friendly, and cost-effective solutions are continuously needed to reduce the burden of fungal contamination and to improve the safety of food and agricultural products.

In sub-Saharan Africa, including Côte d'Ivoire, there is long-standing indigenous knowledge of using native plant extracts as an effective solution against plant pests and pathogens (8–13). Several aromatic plants grown in Côte d'Ivoire have been described to possess medicinal, insecticidal, and antimicrobial effects that were used historically by local communities to control post-harvest contamination of food crops (14,15). Traditionally, farmers would deploy a crude extract of aromatic plants in storage granaries to extend the shelf life of cereal crops by preventing fungal contamination and repelling insects, rodents, and pests. These plants include *Azadirachta indica* Juss (Neem tree), *Cymbopogon citratus* (DC ex Nees) Stapf. (lemongrass), *Allium sativum* L. (garlic), and *Capsicum frutescens* L. (wild chili pepper), that have been previously reported to have antifungal and antibacterial activities against several human and foodborne pathogens (16–18). Additionally, oils extracted from the fresh leaves of these plants possess potent antifungal and antimicrobial activities against *Aspergillus*, *Fusarium,* and *Penicillium* spp. (19,20). However, essential oil extraction is time-consuming, requires specialized equipment, and produces low yields, making the development of oils as fungicides prohibitively expensive for farmers in sub-Saharan countries. Thus, an alternative solution is to explore the ability of an aqueous formulation of these plants to reduce or inhibit fungal contamination of food crops. The use of individual plant extracts offers valuable insights into the specific antifungal mechanism of bioactive metabolites and allows for a more targeted approach against specific microorganisms. However, mixtures of plant extracts have previously

been reported to exhibit combination effects that enhances their bioactive properties (21–24). Thus, recapitulating traditional knowledge and ancestral practices of using native aromatic plant mixtures for the development of a biofungicide could increase the bioactivity against microorganisms and the diversity of microbial targets.

BioCC+ is an aqueous extract of plant mixtures of *Azadirachta indica* Juss (Neem tree), *Cymbopogon citratus* (DC ex Nees) Stapf. (lemongrass), *Allium sativum* L. (garlic), and *Capsicum frutescens* L. (wild chili pepper) developed from traditional knowledge and ancestral practices of using plants to protect agricultural crops. The constituents of BioCC+ are well-known edible plants in West Africa with very low toxicity ranging between 2000 mg/kg body weight for *C. citratus* (lemongrass) and 500 mg/kg body weight for *A. sativum* (garlic) by oral administration (25,26). The objectives of this study were two-fold: first, we employed bioactivity-guided fractionation to compare the efficacy of crude extracts and fractions of BioCC+ against two important cereal pathogens, *F. graminearum* and *F. verticillioides* through *in vitro* antifungal assays. Then, we used untargeted metabolomics and biochemometric approaches to identify bioactive metabolites from the crude extracts and fractions to potentially explain observed antifungal effects against *Fusarium* spp. Outcomes from this study could provide insights into the antifungal efficacy of BioCC+ and help local researchers and farmers with further research and development of this prototype fungicide for the control of fungal contamination in cereal crops.

Methods

Development and preparation of BioCC+

The polyherbal formulation BioCC+ is an aqueous solution developed by a research team at Jean Lorougnon Guede University, Côte d'Ivoire. BioCC+ is a mixture of plant material harvested from local medicinal plants: leaves of *A. indica* and *C. citratus*, bulbs of *A. sativum*,

and fruits of *C. frutescens*. Plant material was macerated together in distilled water for 48 hours at room temperature (22-23°C) with shaking, filtered, and oven dried for 4 days at 40°C. The dry extract, herein referred to as native crude extract (NCE), was packaged in plastic jars and stored at 4°C until use.

Fusarium strains used in this study

Fusarium graminearum (CB-CS-5) and *F. verticillioides* (61-MS-CS) were provided by Dr. Gretchen Kuldau (The Pennsylvania State University) for this study. Fungal spores were obtained by plating *Fusarium* isolates onto sporulation medium, Synthetic Nutrient-poor Agar (SNA; 1 g/L KH_2PO_4, 1 g/L KNO_3, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.5 g/L KCl, 0.2 g/L glucose, 0.2 g/L saccharose, 20 g/L agar). Inoculated plates were incubated at 28 ± 2°C for 7 days. Spores were harvested using 0.05% Tween-20 and stored in 25% glycerol at -80°C with a concentration of 10^6 spores/mL. Identity of fungal strains were confirmed by PCR amplification followed by Sanger sequencing of the ITS1/ITS4 region using primers (ITS1F: 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4: 5′-TCCTCCGCTTATTGATATGC-3′) (27).

Antifungal assay

Antifungal activity assays were conducted on *F. graminearum* and *F. verticillioides* in 24-well microplates using a range of concentrations for NCE, chemical extracts, and fractions (detailed description below) dissolved in DIFCO™ Potato Dextrose Agar (PDA) (BD, Franklin Lakes, NJ, USA), to a final volume of 2 mL/well containing fungal growth medium with test compounds. DMSO (2%) (Sigma-Aldrich, St. Louis, MO, USA) was used as a vehicle for chemical extracts and fractions. The range of concentrations tested was guided by antifungal efficacy (percent growth inhibition of test condition compared to control). Therefore, the range

for NCE, MeOHe, and H_2Oe were 3.0 to 25.0 mg/mL, DCMe was between 0.8 mg/mL and 6.0 mg/mL, and fractions were evaluated between 0.2 mg/mL and 2.0 mg/mL.

A 1 µL spore suspension containing 10^5 spores/mL (equivalent to 100 spores/well) of each *Fusarium* strain was center inoculated in each test well and incubated at 30°C for 3 days. For each test compound and control, four wells were used, and the experiment was repeated twice (two independent biological experiments) for each concentration range (total, n = 8). Controls for each condition included an uninoculated PDA, PDA with no antifungal treatment inoculated with fungal isolates, and PDA + DMSO vehicle inoculated with fungi. Fungal colony diameter was used to measure growth inhibition, and the minimum inhibitory concentration (MIC) was defined on day 3 as the lowest concentration that completely inhibits fungal growth within the range of tested concentrations and conditions. Percent growth inhibition was calculated using the following formula: Growth Inhibition (%) = [(Dc - Dt) / Dc] × 100, where: Dc = colony diameter of the control and Dt = colony diameter of the treatment.

Bioactivity-guided fractionation of BioCC+

The organic solvents dichloromethane (DCM) and methanol (MeOH) used for chemical extractions were ACS-grade and purchased from Sigma-Aldrich. Native crude extracts were sequentially extracted with DCM, MeOH, and distilled water (dH_2O) in order of increasing polarity (**Figure S1**). Initially, 20 g of NCE was macerated in 250 mL DCM for 48 hours while stirring at room temperature (22-23°C). Then, the macerate was filtered and evaporated to obtain the DCM extract (DCMe). The residue was subsequently extracted using 250 mL MeOH to obtain a MeOH extract (MeOHe). Finally, a water extract (H_2Oe) was obtained by the addition of 250 mL dH_2O from the residue of methanol extraction. Each extract was tested for antifungal activity.

Based on the observed growth inhibition of DCMe against *Fusarium* strains, DCMe was selected for further separation using silica gel column chromatography using the following mixtures of hexane:ethyl acetate as the mobile phase: 100:0 for F1, 75:25 for F2, 50:50 for F3, 25:75 for F4, and 0:100 for F5, as well as 100% methanol for F6 (**Table S1**). The solvents were removed from each fraction using a rotary evaporator. The residue of each fraction was weighed and stored at 4°C until use. Of the 6 fractions, 5 with the most distinct TLC profiles (F2, F3, F4, F5 and F6) were selected for antifungal activity assays against *Fusarium* strains and subsequent analysis by Liquid Chromatography-Mass Spectrometry (LC-MS).

Liquid Chromatography Mass Spectrometry Analysis

Native crude extract, DCMe, and fractions (F2, F3, F4, F5, F6) were selected for analysis using a Vanquish UHPLC system coupled to an Exploris 120 mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with a heated electrospray ionization (HESI) source at the Metabolomics Core Facility at The Pennsylvania State University. Separation was achieved on a Waters Acquity CSH C18 column (2.1 × 100 mm, 1.7 μm particle size), maintained at 40°C. The mobile phase consisted of solvent A (LC-MS grade water with 0.1% formic acid) and solvent B (LC-MS grade acetonitrile with 0.1% formic acid). The gradient elution program was as follows: 0–2 min at 3% B, 12 min at 40% B, 15 min at 85% B, 21 min at 100% B, and 25–30 min at 3% B. The flow rate was 0.25 mL/min with a 2 μL injection volume. Data were acquired in both positive and negative ionization modes at a resolution of 120,000 and over a scan range of 80–1000 Da. Ion spray voltages were set at 4,000 V for positive mode and -2600 V for negative mode. Additional parameters included a sheath gas pressure of 35 Arb, auxiliary gas pressure of 10 Arb, sweep gas pressure of 1 Arb, a vaporizer temperature of 150°C, and an ion transfer tube temperature of 325°C.

LC-MS Data Processing

Raw data processing

Raw MS files (.raw) were processed using MS-DIAL version 5 for automatic peak detection using parameters for Exploris 120 (28). Peak detection parameters included a minimum peak height of 10^4 amplitude, an MS tolerance of 0.005 Da, and a retention time range of 0-30 min. Using MS-DIAL, peak spectra were detected, deconvoluted, annotated, and aligned. To ensure accurate peak detection, peaks were filtered against blanks, with tentative annotations assigned to all tested features using the MS-DIAL library files under the following criteria: a minimum MS1 spectrum match of 65% and at least 3 MS2 spectra match. The resulting aligned peak table, containing mass to charge (*m/z*) values, MS2 spectra, RT, peak areas, and tentative annotation, was exported into an Excel file (.xls) (deposited into ScholarSphere data repository (1). Metabolites detected in the blank samples were subtracted from the tested samples. The characterization of F1 was not included in antifungal activity assays and biochemometric analysis due to its low extracted mass.

Chemometric and Biochemometric Analysis of Compounds Driving Antifungal Activity

Multivariate data analysis was used for the characterization of potential antifungal compounds driving the observed growth inhibition of BioCC+ against *F. graminearum* and *F. verticillioides*. Using the annotated features and peak areas, Partial Least Squares Discriminant Analysis (PLS-DA) was performed to discriminate metabolomic profiles of F2-F6 using MetaboAnalyst 6.0 (29). Growth inhibition data was concatenated to the metabolomic dataset for the five fractions (F2, F3, F4, F5, and F6) to identify potential bioactive metabolites(1). The concentration of fractions against *F. graminearum* was selected as follows: 1.0 mg/mL was used for F2, F3, F5, and F6, while 0.8 mg/mL was used for F4. Although two fungal strains were used for antifungal activity assays, we focused on *F. graminearum* for biochemometrics

as this isolate exhibited the highest susceptibility against the tested fractions when compared to *F. verticillioides*. The selected concentration of F4 was lower (0.8 mg/mL) than the other fractions due to a lack of overlap in the range of concentrations tested when compared to the other fractions. The biochemometrics dataset was used to perform a Partial Least Square Regression (PLS-R) via SIRIUS version 13.5 (Pattern Recognition Systems AS, Bergen, Norway). The PLS model was developed with internal cross-validation performed over 100 iterations using a significance threshold of 0.05.

Identification of key antifungal compounds

Putative lead antifungal compounds in tested fractions were identified using two multivariate models, focusing on features with high peak intensities and strong correlations with percent fungal growth inhibition. A feature is defined as a unique *m/z*_RT combination. The selection of features with the highest peak areas in tested fractions with the highest percent growth inhibition was informed by the Variable Importance in Projection (VIP) scores from PLS-DA and by the covariance values from the S-plot generated by PLS-R model. Concurrently, features with strong correlations between peak areas and percent growth inhibition were prioritized based on correlation coefficients observed in the PLS-R S-plot and Selectivity Ratio (SR) values. The importance of features from each model output was ranked as outlined in **Table S2**. Finally, the top 10 features across both models were combined to generate a list of putative metabolites driving antifungal activity observed in the tested fractions derived from the DCMe of BioCC+. The metabolomic profiling workflow of lead antifungal compounds is summarized in **Figure S2**.

Results and Discussions

Comparing the antifungal efficacy between native crude extract, chemical extracts, and fractions of BioCC+ against *Fusarium* species

Native crude extracts of BioCC+ demonstrated dose-dependent antifungal activity against *Fusarium* strains. Complete growth inhibition (100%) was observed at 12.5 mg/mL with *F. graminearum* and 25.0 mg/mL with *F. verticillioides*, indicating that *F. graminearum* was more susceptible to BioCC+ NCE compared to *F. verticillioides* (**Figure 1A**). Solvent partitions of the NCE were used to isolate bioactive compounds from the aqueous BioCC+ NCE. Antifungal activity assays demonstrate that the DCMe exhibited strong, dose-dependent antifungal activity, achieving 100% growth inhibition of *F. graminearum* at 1.6 mg/mL and *F. verticillioides* at 6.0 mg/mL (**Figure 1B**). In contrast, neither the H_2Oe nor MeOHe achieved complete growth inhibition of both *Fusarium* strains within the tested concentration range (**Figure 1C and D**).

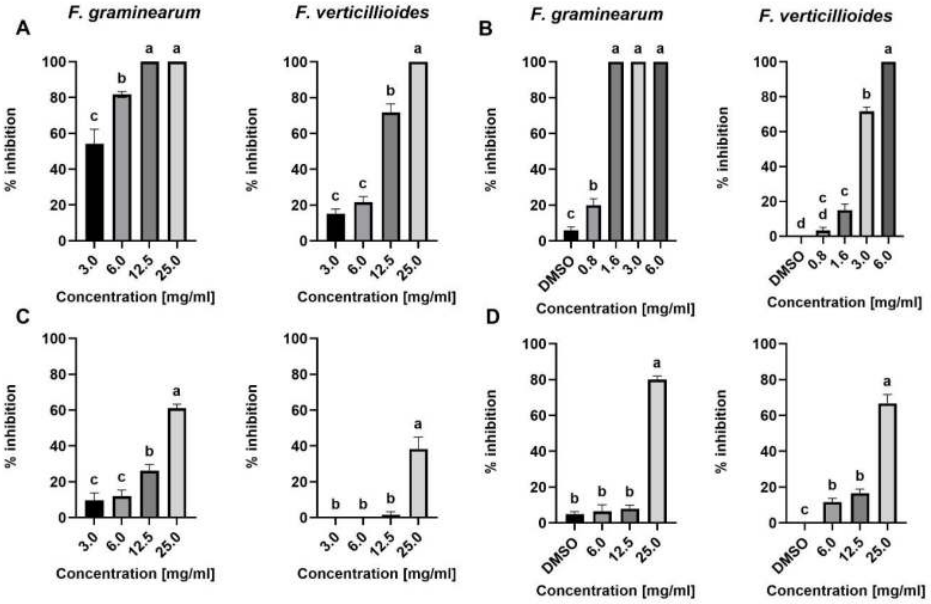
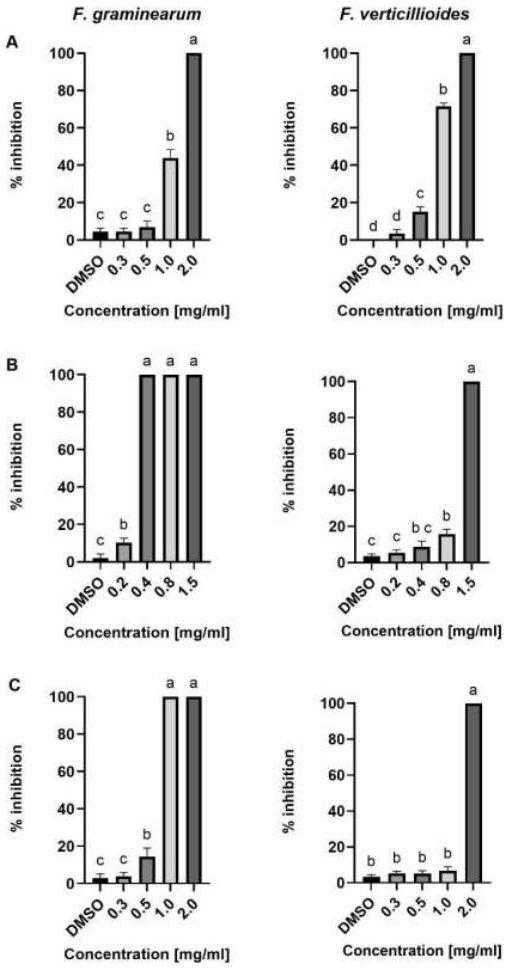


Figure 1 The effect of BioCC+ NCE (**A**), DCMe (**B**), H2Oe (**C**), and MeOHe (**D**) on growth of *F. graminearum* and *F. verticillioides*. Growth was measured by colony diameter after 72 h and presented as % growth inhibition relative to 0 mg/mL control or DMSO vehicle control

(2%) with mean ± SEM (n=8). Letters above bar graphs indicate statistical significance using one-way ANOVA analysis followed by Tukey's multiple comparison test, $p < 0.05$.

To investigate the relationship between specific compounds in the DCMe and their antifungal properties, DCMe was further fractionated using silica gel chromatography resulting in 6 fractions. Among these fractions (F), F3, F4, and F6 exhibited the highest antifungal activity against both *Fusarium* strains (**Figure 2**). We observed complete growth inhibition of *F. graminearum* at 2.0 mg/mL, 0.4 mg/mL, and 1.0 mg/mL for F3, F4, and F6, respectively (**Figure 2**). Similar to antifungal activity of NCE and chemical extracts, *F. verticillioides* was more resistant to the antifungal effects of tested fractions. Fractions F2 and F5 did not demonstrate significant antifungal activity compared to F3, F4, and F6 (**Figure S3**). The MICs for all tested fractions compared to chemical extracts and NCE are summarized in **Table S3**.

While DCM was selected in the present study for its efficiency in recovering medium- to low polarity metabolites to help explain which metabolites drive antifungal activity, its volatility and environmental toxicity limit agricultural application. The DCM extract was primarily used for analytical purposes, rather than a formulation intended for field use. From a feasibility standpoint, several strategies can enhance the sustainability and applicability of BioCC+. The crude extract of BioCC+ may be used directly, or the material may be re-extracted or reformulated using alternative solvents such as ethanol, ethyl acetate, or ethanol mixtures, which offer a broad polarity range and are approved for agro-industrial applications (30). Another strategy is to optimize formulation by incorporating the active fractions or purified compounds into biocompatible carriers (e.g., emulsifiable concentrates, microencapsulated or nanoparticle-based systems) that improve stability and delivery for intended use (31). Collectively, these strategies would enable translation of the current antifungal leads from analytical extracts to environmentally sustainable BioCC+-derived biocontrol formulations suitable for agricultural deployment.



289

290 **Figure 2** Effect of the most bioactive fractions on growth of *F. graminearum* (left column)
291 and *F. verticillioides* (right column). Growth of *Fusarium* spp. was measured using colony
292 diameter and presented as % growth inhibition relative to the DMSO vehicle control (2%) for
293 F3 (**panel A**), F4 (**panel B**), and F6 (**panel C**). Bar graphs represent mean ± SEM of % growth
294 inhibition (n=8). Letters above bar graphs indicate statistical significance using one-way
295 ANOVA analysis followed by Tukey's multiple comparison test, $p < 0.05$.

296 Overall, F4 had the highest antifungal efficacy against *F. graminearum* (MIC =
297 0.4mg/mL) compared to *F. verticillioides* (MIC = 1.5mg/mL). The observed susceptibility of

298 *F. graminearum* compared to *F. verticillioides* could be attributed to genetic differences
299 between the fungal isolates. Notably, *F. verticillioides* has been reported to grow more rapidly
300 and produce more toxic secondary metabolites at 30°C than *F. graminearum* (32). A relevant
301 future study would be to test the antifungal efficacy of BioCC+ and its chemical extracts and
302 fractions with *Fusarium* isolates grown at various temperatures and on diverse growth media.
303 This could provide insights into mechanistic differences of growth inhibition between *F.*
304 *graminearum* and *F. verticillioides*.
305
306 **Metabolomic profiling of DCM fractions derived from BioCC+**
307 Using MS-DIAL, raw chromatograms from positive and negative modes for all 6
308 fractions resulted in 22,416 and 11,212 identified features (defined as unique *m/z*_RT pairs),
309 respectively (1). The MS-DIAL spectral library files were used to tentatively annotate features,
310 where 59% of detected features were annotated in positive mode while 40% of the detected
311 features were annotated in the negative mode. Here, we focused on annotated features for
312 downstream metabolomic characterization and identification of lead antifungal compounds
313 driving observed growth inhibition of the most bioactive fractions.
314 To assess similarities and differences in chemical profiles (chemometrics) of bioactive
315 fractions, a Principal Component Analysis (PCA) was conducted using the peak areas of the
316 annotated features. The top two components of the PCA accounted for 74.6% and 67% of the
317 total variance in positive and negative modes. For both modes, F2 and F3 appear to cluster
318 together along PC1, suggesting similarities in their respective chemical profiles. All other
319 fractions were distinctively different from each other, with F4 having the highest distinction in
320 positive ion mode, while F6 had the highest distinction along PC1 (**Figure 3**). The clear
321 metabolomic separation of F4 and F6 from other fractions aligns with their observed high

percent growth inhibitions, suggesting variation in their chemical composition could help explain observed antifungal properties.

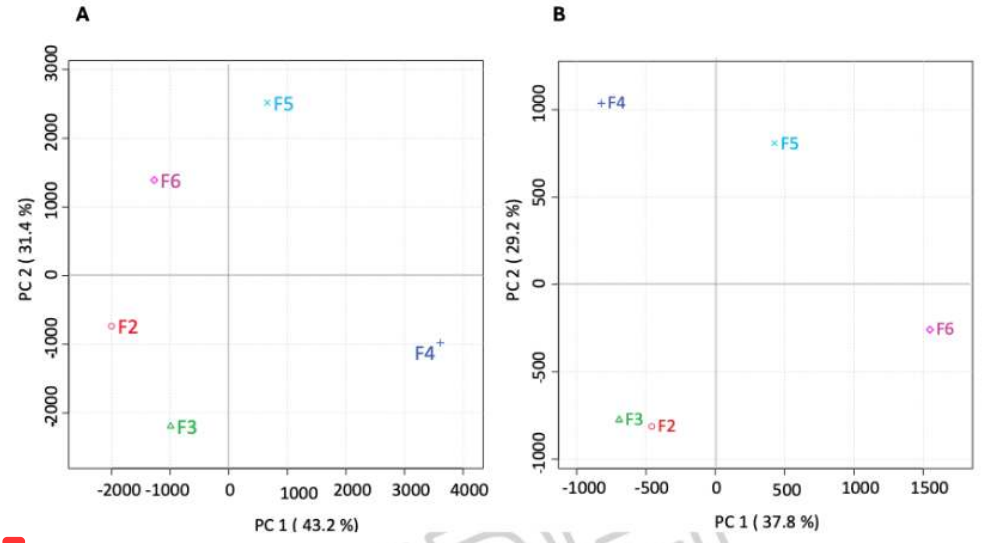


Figure 3 Principal component analysis (PCA) scores plot of the metabolomic profiles from fractions (F2, F3, F4, F5, and F6) in positive **(A)** and negative mode **(B)** using Metaboanalyst 6.0.

Characterization of lead antifungal compounds from DCM fractions of BioCC+

To identify the bioactive compounds driving fungal growth inhibition across fractions, a combination of two multivariate analyses, Partial Least Squares Discriminant Analysis (PLS-DA) and Partial Least Squares Regression (PLS-R), highlighted key antifungal features based on criteria defined in **Table S2**. PLS-DA (Permutation testing p<0.0005 (0/2000 permutations), identified features with Variable Importance in Projection (VIP) scores above one (VIP>1.0), and the highest mean peak areas (dark, red-colored cubes) in F4 were identified as potential lead antifungal compounds with strong discriminatory power (p<0.05) (**Figure 4**). From the PLS-DA VIP score plot with the 20 highest VIP score features, all 20 features in positive mode and 2 in negative mode were identified as potential lead antifungal compounds.

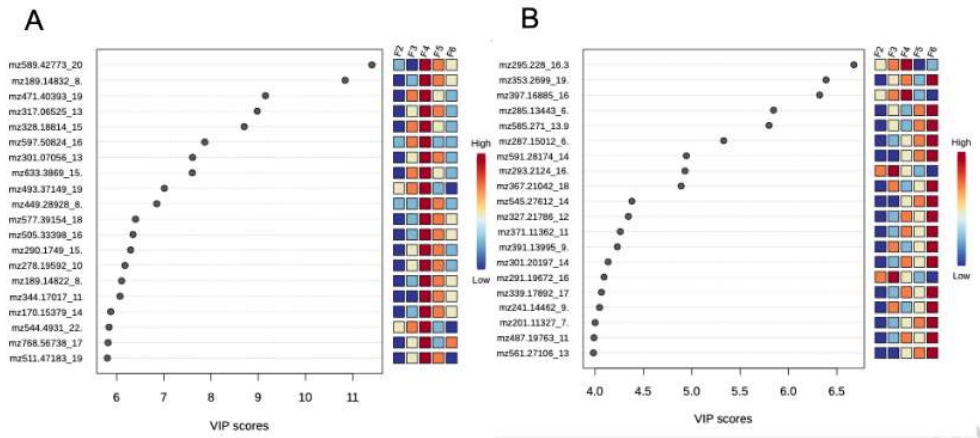


Figure 4 The variable importance in projection (VIP) score plots from Partial Least Squares Discriminant Analysis (PLS-DA) with the top 20 highest VIP features identified in the bioactive fractions F2 through F6, both in the (A) positive and (B) negative mode. The y-axis represents annotated features, and the x-axis shows VIP scores. Dark blue to dark red boxes represent metabolite peak areas ranging from lowest to highest, respectively, with yellow indicating intermediate peak areas.

To provide complementary insight into bioactive compounds, a partial least squares regression (PLS-R) model was developed by integrating metabolomic data with antifungal activity, measured as percent growth inhibition. This biochemometric approach enables the identification of metabolic features that correlate with antifungal activity by linking variation in metabolite profiles across fractions to bioactivity responses. In doing so, this could provide mechanistic insight into the association of specific chemical classes, such as alkaloids, flavonoids, terpenoids, and fatty acids, with our observed antifungal effects (33). The top two PLS-R components in the positive mode explained 57.03% and 99.45% of the variance in independent and dependent variables, respectively (**Figure 5**). In the negative mode, the top two components captured 58.05% and 99.97% of the variance in independent and dependent variables. PLS-R score plots revealed clustering patterns similar to PCA results, showing close

spatial relationships between F2 and F3, as well as F4 and F6. The closer proximity between F4 and F6 observed in the PLS-R scores plot, compared to the PCA plot (**Figure 3**), is likely attributed to the antifungal efficacy against *F. graminearum* at tested concentrations of 0.8 mg/mL (F4) and 1.0 mg/mL (F6) (**Figure 5**). The S-plot and Selectivity Ratio (SR) analyses from the PLS-R model identified marker compounds closely associated to the growth inhibition observed by antifungal assays. These target projection techniques enhance biochemometric applications by improving effective bioactive compound discovery while minimizing the peak area only bias of other metabolomics analyses, such as PLS-DA (34,35). Targeted projections enhance biochemometric applications by improving bioactive compound discovery while minimizing peak area bias compared to other metabolomics analyses such as PLS-DA(34,35).

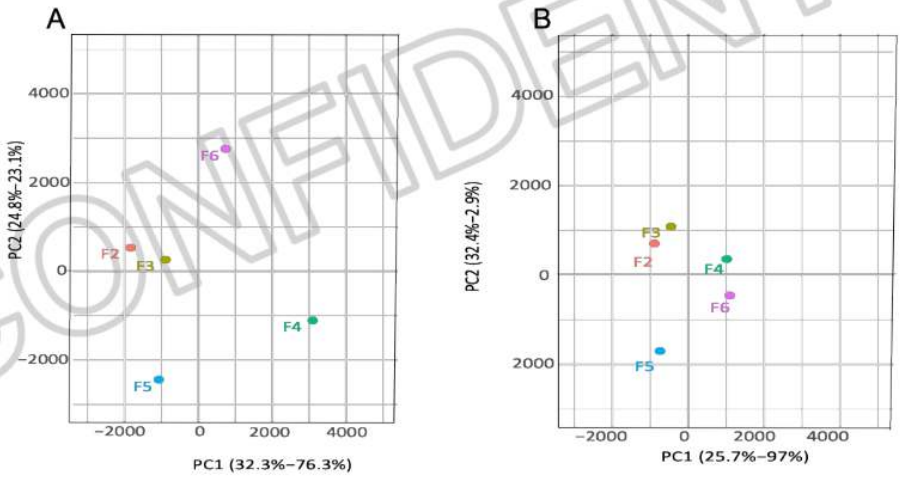


Figure 5 Score plots for the first two partial least squares regression (PLS-R) components (% independent variable - % dependent variable) in positive **(A)** and negative **(B)** mode. Independent variables were the peak areas of the mass spectrometry features, and the dependent variable was percentage growth inhibition.

We combined the top 10 features from PLS-DA VIP, PLS-R Covariance, PLS-R Correlation, and PLS-R SR analyses to provide a chemometric and biochemometric consensus

approach to identify putative antifungal compounds in BioCC+. This integration resulted in the identification of overlapping features (**Figure S4**) and a list of 67 tentatively identified lead antifungal features in positive (31) and negative (36) modes (1). Features identified from model outcomes with similar statistical foundations showed notable overlap, showing the potential bias associated with relying on individual methods. For instance, PLS-DA and PLS-R covariance analyses shared five overlapping features in the positive mode and two in the negative mode (**Figure S4**). This overlap arises because both PLS-R covariance and PLS-DA VIP scores prioritize features based on their quantitative contribution (e.g., peak area magnitude) to the response variable within the model. To mitigate this inherent bias, the incorporated metrics of PLS-R correlation and SR had four overlapping features in positive and two in the negative ion mode **(Figure S4)**. PLS-R correlation and SR facilitate the identification of features with consistent predictive power, emphasizing variables whose trends are strongly aligned with the response rather than solely their magnitude. These findings underscore the importance of integrating multiple models to enhance the accuracy and reliability of identifying bioactive compounds.

To increase confidence in the reported compounds, lead antifungal candidates identified were cross-referenced with compounds identified through GNPS. In addition, their mass fragmentation spectra were further matched using the Mass Bank of North America (MONA) database to generate a final super list of 26 putatively identified lead antifungal compounds (**Tables 1 and 2**). Synthetic compounds and primary metabolites were excluded. Previous literature identified 11 out of 26 metabolites with established antifungal properties that were categorized into flavonoids, alkaloids, terpenoids, polyketides, and hydroxy- and oxy-polyunsaturated fatty acids (**Tables 1 and 2**). Many of these classes are known to exhibit antioxidant, anticancer, antibacterial, and antifungal properties, emphasizing the likelihood that

the putative identified compounds are the lead antifungal agents in the studied fractions (36–39).

Among the tentatively identified compounds was capsaicin, an aromatic fatty amide and the major bioactive component of *C. frutescens* that is known for its antifungal activity against various plant fungal pathogens (40). Another was chrysoeriol, which at 1,000 ppm was found to inhibit the growth of *F. graminearum* by 20.7% when grown *in vitro* for a week (41). While all 11 compounds have demonstrated antifungal properties, to the best of our knowledge, only capsaicin, chrysoeriol, and 2,4-diacetyl phloroglucinol, have been directly evaluated against *Fusarium* species (26,40–42). Some other compounds include alkaloids (hetisine), as well as metabolites previously identified only in soil microorganisms (e.g., 2,4-diacetylphloroglucinol (2,4 DAPG) from *Pseudomonas* spp). Research suggests that compounds with antibiotic properties produced by endophytic microorganisms, such as 2,4 DAPG from *Pseudomonas fluorescens*, may increase the overall bioactivity observed in plant extracts and plant growth (43,44). In addition, 2 compounds (2,4-dihydroxy-6-methyl-3-[(2E,4E)-3-methyl-5-[(1R,2R,6R)-1,2,6-trimethyl-3-oxocyclohexyl]penta-2,4-dienyl] benzaldehyde and ancistrocladine) were identified to not have any antifungal activity, while 13 other metabolites had not been studied for their antifungal activities (**Tables 1 and 2**). Some of these unknown compounds share structural similarities with bioactive classes such as terpenes, alkaloids, hydroxy polyunsaturated fatty acids, and polyphenols—classes commonly associated with antifungal activity. For example, FA 18:2+2O, an oxidized polyunsaturated fatty acid derivative of linoleic acid, a compound that has been found to exhibit >55% of growth inhibition against multiple plant pathogens, including *Rhizoctonia solani* and *Pythium ultimum* (38).

Biochemometric analysis indicates that F4 and F6 are enriched in multiple classes of chemical compound that associate with bioactivity, including flavonoids (e.g., isorhamnetin, chrysoeriol), phenylpropanoid and lignin derivatives, oxidized polyunsaturated fatty acids (e.g.,

9-HODE), short- and medium-chain fatty acids (e.g., lauric acid), polyketides (e.g., 2,4-diacetylphloroglucinol), and select terpenoids. These classes have well-documented antifungal mechanisms such as membrane disruption and increased permeability, disruption of ergosterol biosynthesis or ergosterol-dependent membrane architecture, and enzyme inhibition or induction of oxidative stress (45–47). The co-occurrence of these chemotypes within F4 and F6 likely results in additive or synergistic effects, providing a plausible explanation for their enhanced bioactivity relative to other fractions. The putative identification of these compounds underscores the need for further validation of their antifungal properties. Follow-up studies to definitively annotate these unknown metabolites by testing the bioactivity using chemical standards, targeted nuclear magnetic resonance spectroscopy, and targeted mechanism of action studies are essential for determining their potential applications in combating fungal pathogens.

Using biochemometrics that combines antifungal activity and metabolomic profiling, our study demonstrates that plant mixtures of BioCC+, a formulation traditionally developed based on ancestral practices in Côte d'Ivoire is effective in inhibiting the growth of two major fungal pathogens, *F. graminearum* and *F. verticillioides*. Integration of bioactivity assays with chemometric data using multiple multivariate data analysis models help explain the observed efficiency of BioCC+ components, facilitating the discovery of novel compounds for development of new biofungicides. Outcomes from this study could provide insights into sourcing local plant extracts to further improve the strength and specificity of the prototype biofungicide by integrating antifungal efficacy with metabolite fingerprinting.

Conflicts of interest

The author(s) declare that there are no conflicts of interest.

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Author contributions

Conceptualization: EKK, JDL, JW; Data curation: EKK, ZI, NVV, JW; Formal analysis: EKK, ZI, NVV, JW; Funding acquisition: EKK, JDL, JKK, JW; Investigation: EKK, ZI, NVV; Methodology: EKK, ZI, NVV, JDL, JJK, JW; Project administration: EKK, JW; Resources: EKK, JDL, JJK, JW; Supervision: NVV, JDL, JJK, JW; Visualization: ZI, NVV, JJK, JW; Writing – original draft: EKK, ZI, JW; Writing – review & editing: EKK, ZI, NVV, JDL, JJK, JW.

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471 **Table 1** Putative lead antifungal compound based on chemometrics and biochemometrics profiling of fractions in positive ion mode

Model output	Feature (m/z_RT)	Adduct type	Putative ID	MS/MS spectrum	Class	Reported Antifungal Activity[a]	Ref.
Cov+DA	mz597.50824_16.6	M+H]+	Peltatoside	66.34396:37520 69.07000:112665 81.07030:36180 83.08574:91077 85.40976:35060 97.10139:78728 133.10022:36222 182.98813:37959 197.15578:39870 225.99864:37343 236.15514:32128 245.22656:102001 263.23639:195931 281.24805:319329 366.71497:34229	Flavonoid	Yes	(48)
Cov+DA	mz633.3869_15.22	[M+H]+	(1S,2R,4aS,6aR,6bR,10S,12aR,14bS)-1,2,6b,9,9,12a-hexamethyl-10-[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-methyloxan-2-yl]oxy-2,3,4,5,6,6a,7,8,8a,10,11,12,13,14b-tetradecahydro-1H-picene-4a,6a-dicarboxylic acid	71.19155:10070 75.17818:11865 84.60880:9755 124.54491:9972 148.60321:10914 206.42096:10565 233.98193:9604 271.73001:10272 328.18839:5332368 329.18921:20933 433.91739:10685 607.53796:13719 633.39026:85750	Terpene	Unknown	
Cov	mz196.09656_9.919	[M+H]+	N-Methyltyrosine	90.94788:8232	Amino-acid derivative	Unknown	
Cov	mz411.20633_15.08	[M+Na]+	Ingenol-5,20-acetonide	50.15414:4409 160.27075:4058 181.05844:3768 188.01779:3786 212.04924:8381 229.24011:3709 230.90405:4060 241.05238:7066 259.06253:10971 281.08228:6026 286.30154:4271 352.17081:8231 370.18082:20674 388.19254:12461 411.19189:4396	Diterpene ester	Unknown	
Cov	mz430.20087_8.671	[M+Na]+	Ancistrocladine	135.11742:7304 166.00751:28771 184.01813:54325 199.04164:286501 224.04944:155203 242.06003:172094 292.11215:48661 368.16473:111891	Naphthylisoquinoline alkaloids	No	(49)

DA	mz317.06525_13.592	[M+H]+	Isorhamnetin[bc]	150.76413:3978 153.01820:8523 210.00169:3797 214.32657:3427 257.04398:4971 274.04718:6999 285.03943:8113 302.04221:44422 317.06564:397940 317.21237:12658	Flavonoid	Yes	(50)
DA	mz301.07056_13.303	[M+H]+	Chrysoeriol[b]	81.19906:4887 100.50779:5300 131.07025:10691 211.19273:7084 225.88417:6758 258.05319:27447 286.04712:527681 301.07053:3113106	Flavonoid	Yes	(41)
DA	mz493.37149_19.85	[M+Na]+	Ocotillol	81.06995:18090	Terpene	Unknown	
DA	mz449.28928_8.358	[M+H]+	[(2E,6Z,10Z)-7,11-bis(acetyloxymethyl)-3,15-dimethylhexadeca-2,6,10,14-tetraenyl] acetate	95.08549:82849 123.08047:63146 151.14827:41281	Polyunsaturated fatty acid esters	Unknown	

[a] Column 'Antifungal' refers to annotated antifungal activity based on published literature: 'Yes' denotes published literature with demonstrated antifungal activity of the tentatively identified compound through *in vitro* or *in vivo* studies; 'No' denotes no antifungal efficacy of the tentatively identified compound against at the highest tested concentrations; 'Unknown' denotes no studies were found that specifically tested the compound for its antifungal properties

[b] refers to compounds whose MSMS spectra has been identified with MONA library in addition to MS-DIAL

[c] refers to compounds whose names, m/z and RT have been putatively identified using GNPS library in addition to MS-DIAL

'null' refers to compounds with no found fragment ions of the parent compound using MS-DIAL

Model output (A) refers to the model output from which the compound was identified in the top ten lead antifungal compound

m/z_RT refers to mass to change_ Retention time of the compounds

MS/MS spectrum refers to fragment ions of the tentatively identified compounds

Table 2 Putative lead antifungal compound based on chemometrics and biochemometrics profiling of fractions in negative ion mode

Model output	Compound (m/z_RT)	Adduct type	Putative ID	Assigned MS/MS spectrum	Class	Reported Antifungal Activity[a]	Ref.
Corr+Cov	mz375.14499_11.433	[M-H]-	4-[4-[hydroxy-(4-hydroxy-3-methoxyphenyl)methyl]-3-(hydroxymethyl)oxolan-2-yl]-2-methoxyphenol	59.01411:10958 80.68203:3933 91.47937:4642 112.98581:8676 114.98586:3957 136.05322:8638 151.07657:4041 168.65164:3663 194.05852:5911 195.06659:62552 209.08124:32360 375.14505:57780	Phenylpropanoids	Unknown	
Cov +DA	mz397.16885_16.602	[M-H]-	(2R,3S,4S,9R,12R,13R,14S,15R,16R,17S)-3,4,12,13,15,16-hexahydroxy-2,6,14,17-tetramethyl-10-oxatetracyclo[7.7.1.0^{2},^{7}.0^{13},^{17}]heptadec-6-en-11-one	59.84045:4068 64.22029:4444 70.24025:4126 84.93886:6867 99.92660:5784 100.93346:22426 116.92852:44148 158.15057:3686 223.14903:4543 268.98553:6482 281.05222:4791 315.14337:5485 317.70523:4543 335.16519:4782 343.13608:5584 353.18021:9809 356.98959:5398 361.14725:20883 377.00476:5787 379.15836:15421 397.00284:29044 397.16879:123977	Terpenoid	Unknown	
Cov +DA	mz357.13425_12.727	[M-H]-	Matairesinol[bc]	83.01396:75522 136.05246:33237	Phenylpropanoids /Lignans	Yes	(51)
Cov	mz369.26483_16.909	[M-H]-	2,4-dihydroxy-6-methyl-3-[(2E,4E)-3-methyl-5-[(1R,2R,6R)-1,2,6-trimethyl-3-oxocyclohexyl]penta-2,4-dienyl]benzaldehyde	89.02449:42134 185.11845:24373	Phenols	No	(52)
Cov	mz353.2699_19.978	[M-H]-	5-[5-hydroxy-3-(hydroxymethyl)pentyl]-8a-(hydroxymethyl)-5,6-dimethyl-3,4,4a,6,7,8-hexahydronaphthalene-1-carboxylic acid [c]	89.02448:32563	Diterpenoid	Unknown	
Cov	mz339.17892_17.137	[M-H]-	[5-acetyloxy-3-(hydroxymethyl)-2-oxo-6-	89.02448:78774	Monoterpenoid	Unknown	

			propan-2-ylcyclohex-3-en-1-yl] 3-methylpentanoate				
Cov	mz209.04565_8.456	[M-H]-	4-diacetylphloroglucinol[b]	61.30024:3992 65.07722:4267 75.60643:3296 91.00390:3964 95.01394:7207 109.02916:4413 120.88881:3934 123.04543:9308 126.50152:4194 137.02432:10121 137.06058:12715 146.93240:4177 165.01938:238186 165.05537:18476 191.03572:5105 209.04570:653646	Polyketides	Yes	(42)
Cov	mz285.13443_6.378	[M-H]-	Methyl 2-ethyl-4-[(3R,4R,5S)-5-hydroxy-4,5-dimethyl-2-oxooxolan-3-yl]-2-methyl-3-oxobutanoate	56.39479:3102 56.71626:3556 65.52523:3807 285.13388:29690	Polyketides	Unknown	
Cov	mz327.21786_12.981	[M-H]-	FA 18:2+3O[b]	57.03474:5037 74.11918:4863 85.02963:31016 97.06573:4362 97.32687:4325 137.09737:12057 171.10281:80794 171.12018:6798 179.88539:4385 183.13921:4400 211.13365:34522 229.14487:28635 239.12941:6836 291.19577:10441 327.21799:415282	Oxidized polyunsaturated fatty acid	Yes	(38)
Cov	mz199.17038_17.189	[M-H]-	Lauric acid	49.24802:3856 55.50630:4399 61.64085:4321 65.04016:3841 178.99438:5959 198.98123:4567 199.00192:7005 199.17043:1604646	Saturated fatty acid	Yes	(53,54)
DA	mz295.228_16.326	[M-H]-	9-HODE[b]	50.82198:5534 55.26590:6084 67.32764:5731 81.36312:5644 107.99917:6258 123.11747:10507 124.80696:6093 139.11284:18179 155.10782:53702 171.10277:126629 183.10269:451900 185.11726:11253 195.14029:6119 230.98615:6708 250.91420:5776 254.98665:24215 274.99130:9692 277.21744:350730 294.98956:17190 295.22781:2167905	Unsaturated fatty acid	Yes, but it might increase mycotoxin production	(38,55)
DA	mz397.22098_15.11	[M-H]-	Mitragynine	223.13408:23633 293.21118:18238 317.20999:34115	Monoterpenoid indole alkaloid	Unknown	

DA	mz304.19202_15.224	[M-H]-	Capsaicin[bc]	53.37697:4025 55.02234:3892 58.02984:205983 96.99971:3558 141.37459:4200 168.13934:775574 221.16344:4213 265.28772:4106 304.19193:284458	Fatty amide	Yes	(40)
DA	mz311.22299_15.48	[M-H]-	FA 18:2+2O[b]	50.78807:4374 63.02858:3977 71.37285:3869 139.27464:4018 147.54845:3871 204.99005:7001 225.99971:4359 230.98817:5150 247.00117:5038 250.99344:10982 270.97729:5114 290.98758:11653 293.21277:72672 311.01227:29249 311.17654:9735 311.22281:148921	Hydroxy Polyunsaturated Fatty acid	Derivative of Linoleic acid which is known to have antifungal properties	(38)
Corr	mz374.19757_14.196	[M+HCOO]-	Hetisine[c]	null	Terpenoid alkaloid	Unknown	
Corr	mz213.14981_15.258	[M-H]-	FA 12:1+1O	59.01390:81819 59.01745:4655 106.65736:3653 118.92508:4075 144.99080:4984 146.64877:4034 173.00233:5277 192.99039:30508 193.00891:5229 212.99783:16244 213.14986:59101	Hydroxy Unsaturated fatty acid	Yes	(38)
Corr	mz285.02805_10.765	[M-H]-	Kaempferol[c]	null	Flavonol	Yes	(37,56)

[a]Column 'Antifungal' refers to annotated antifungal activity based on published literature: 'Yes' denotes published literature with demonstrated antifungal activity of the tentatively identified compound through *in vitro* or *in vivo* studies; 'No' denotes no antifungal efficacy of the tentatively identified compound against at the highest tested concentrations; 'Unknown' denotes no studies were found that specifically tested the compound for its antifungal properties

[b]refers to compounds that have been tentatively identified with MONA in addition to MS-DIAL

'null' refers to compounds with no found fragment ions of the parent compound using MS-DIAL

[c]refers to compounds whose names, m/z and RT have been putatively identified using GNPS library in addition to MS-DIAL

Model output (A) refers to the model output from which the compound was identified in the top ten lead antifungal compound

m/z_RT refers to mass to change_ Retention time of the compounds

MS/MS spectrum refers to fragment ions of the tentatively identified compounds

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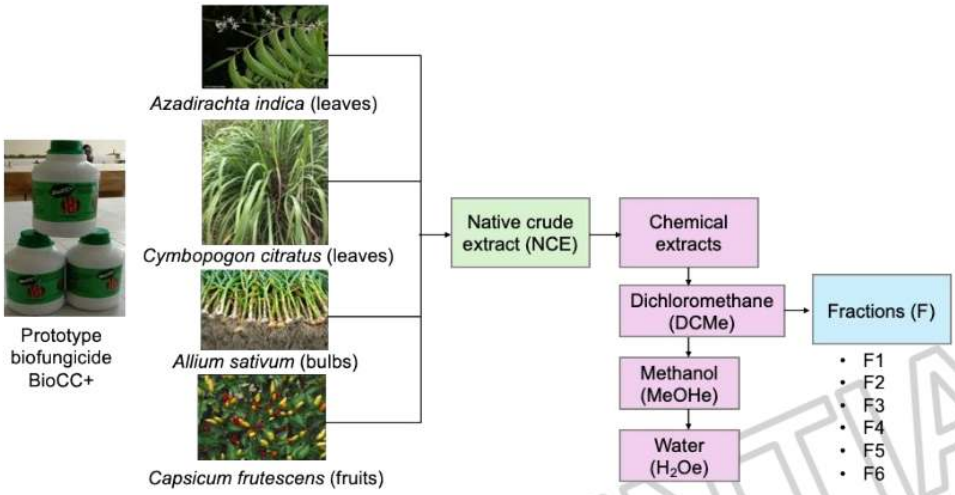

Figure S1 Schematic diagram of extraction and bioactivity-guided fractionation to evaluate the antifungal properties of the polyherbal formulation, BioCC+ against *F. graminearum* and *F. verticillioides*. The green box denotes NCE, three chemical extracts (DCMe, MeOHe, H_2Oe) are indicated in pink boxes, and six fractions (F) are indicated in blue.

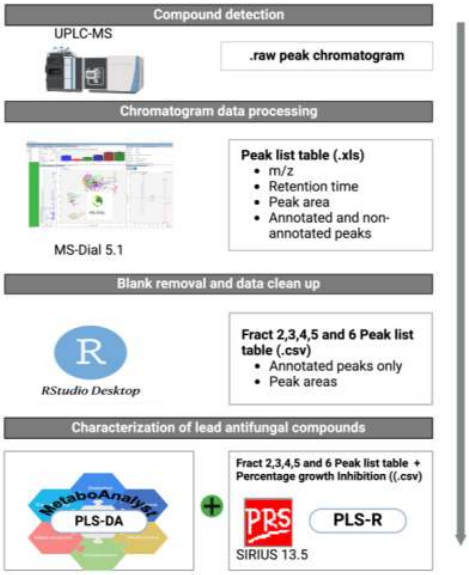

Figure S2 Workflow for the metabolomic identification and characterization of lead antifungal compounds. All samples (NCE, chemical extracts, and fractions) were separated and analyzed using UHPLC-MS. The resulting raw chromatograms were processed in MS-DIAL to extract and quantify features, followed by a tentative annotation. Lead antifungal compounds were identified by integrating chemometric analysis (PLS-DA, using MetaboAnalyst) with biochemometric modeling (PLS-R, using SIRIUS 13.5).

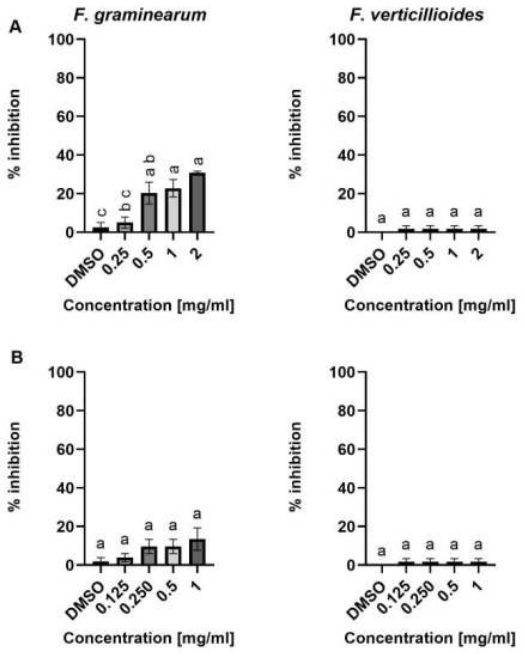

Figure S3 The effect of fractions F2 and F5 on growth of *F. graminearum* (left column) and *F. verticillioides* (right column). Growth of *Fusarium* spp. was measured using colony diameter and presented as % growth inhibition relative to the DMSO vehicle control (2%) for F2 (panel A) and F5 (panel B). The bar graphs represent mean ± SEM of % growth inhibition (n=4). Letters above bar graphs indicate statistical significance using one-way ANOVA analysis followed by Tukey's multiple comparison test, $P<0.05$.

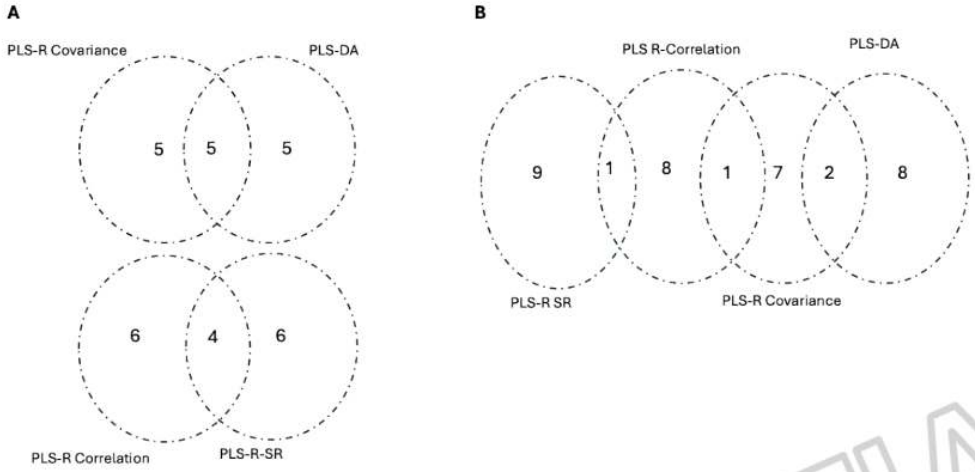

Figure S4 Overlap of key bioactive metabolites identified by PLS-DA and PLS-R in positive (A) and negative (B) ion modes. A consolidated list of highly significant bioactive metabolites was generated for the positive and negative ion modes by combining the top 10 metabolites from each model ranked as most bioactive based on the criteria outlined in **Table S2**.

Table S1 Mobile phase composition used for fractionation of DCM extracts using column chromatography

Mobile phase	Proportions V/V	Fraction	Mass (mg)	Yield (%)
Hexane-Ethyl acetate	100/0	F1	10	1.42
Hexane-Ethyl acetate	75/25	F2	280	40
Hexane-Ethyl acetate	50/50	F3	100	14.2
Hexane-Ethyl acetate	25/75	F4	60	8.57
Hexane-Ethyl acetate	0/100	F5	40	5.72
Methanol	100	F6	210	30

V/V : volume to volume ratio

Table S2 Criteria used to rank features as lead antifungal compounds using PLS-R and PLS-DA

Model	**Outputs of interest from models**	**Criteria for selection of highly important features**
PLS-R	• S-plot correlation[a] • S-plot covariance[b] • SR plot	• >0.9 • >1*10E4 • Highest SR value
PLS-DA	• Variable of Importance Projection (VIP) • Feature's mean peak area in each fraction	• >1.0 • Fract4>Fract1, Fract4>Fract2, Fract4>Fract5, and Fract4>Fract6

[a]**Correlation:** A high positive correlation indicates that the feature's peak area increase aligns with greater fungal growth inhibition

[b]**Covariance:** a high covariance indicates that the feature's large peak area is contributing to the growth inhibition variable, focusing on the magnitude of peak area

Table S3 Summary of MIC values for BioCC+, chemical extracts, and fractions against *Fusarium* spp. grown on PDA at 30°C for 72 h

Species tested	MIC (mg/mL)									
	BioCC +	DCM	MeOH	H2O	F1	F2	F3	F4	F5	F6
F. graminearum	12.5	1.6	>25.0	> 25.0	NT	>2.0	2.0	0.4	>1.0	1.0
F. verticillioides	25.0	6.3	> 25.0	> 25.0	NT	NT	2.0	1.5	NT	2.0

NT : Not tested

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