

## Research Article

# Antibacterial and Antioxidant Properties of Endophytic Fungi Extracts from *Cola acuminata* (Sterculiaceae)

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Received: January 22, 2022; Accepted: February 17, 2022; Published: February 24, 2022

## Introduction

Pneumonia is a disease resulting from a complex set of processes, starting with contact with an infective microorganism and culminating in invasion of the lower respiratory system [1]. Infective microorganisms such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Escherichia coli* are the most incriminated bacteria of this infection [1-3]. The disease burden from pneumonia is a current threat to humanity, causing greater deaths and economic repercussions than any other cause of disease, and it is one of the leading causes of death [1-3]. Worldwide, pneumonia remains the deadliest communicable disease, causing 3.0 million deaths worldwide [4]. In Cameroon, pneumonia was the third leading cause of death, accounting for 22.3% of cases in a study by Tazinya et al. [5] in Bamenda, while 15% and 31% of cases were recorded in Buea for children and post-neonatal deaths, respectively [6].

Once these bacteria reach the alveolar space, macrophages engulf them and trigger signal molecules such as cytokines that recruit other inflammatory cells, such as neutrophils, to the site of infection [7]. One of the effects of the former is the overproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS), such as  $\text{Fe}^{3+}$ ,  $\text{OH}^-$ ,  $\text{ONOO}^-$ ,  $\text{HClO}$ ,  $\text{H}_2\text{O}_2$ , and  $\text{NO}$ , which leads to oxidative stress. The resulting effect of these actions is inflammation of the lung parenchyma, which makes the lining capillaries “leaky”. Consequently, the alveolar sacs are filled with pus and fluid, thereby making breathing difficult and limiting oxygen, leading to pneumonia [2,8].

Disease management measures primarily rely on chemotherapy

## Abstract

Currently, the disease burden from pneumonia remains a major public health problem. In this regard, exploring endophytic fungal extracts from traditionally used plants could be a promising approach in this light. Therefore, this work was conceived with the aim of evaluating the antibacterial and antioxidant activities of two endophytic extracts of *Cola acuminata* against pneumonia-causing bacteria. The identification led to the acquisition of two endophytic fungi: *Trichoderma harzianum* and *Trichoderma afroharzianum*. From ten (10) extracts, two (CAB31P1 and CAF71N2) were active on the tested bacteria, and their MICs ranged from 12.5 to 100 µg/ml. CAF71N2 displayed better antioxidant activity with  $\text{IC}_{50}$  values of 74.75, 12.70 and 5.66 µg/ml for the reducing power of  $\text{Fe}^{3+}$ , NO and OH radical scavenging capacities, respectively. The extracts revealed no cytotoxicity ( $\text{CC}_{50} > 100$  µg/ml) on the two cell lines tested. These results suggest that the endophytic extracts from *Cola acuminata* could serve as a source for the isolation of potent antibacterial and antioxidant compounds for the treatment of pneumonia.

**Keywords:** *Cola acuminata*; Endophytic fungi; Antibacterial; Antioxidant; Pneumonia

using antibiotics such as those from fluoroquinolone and tetracycline classes. Despite their effectiveness, the latter face several secondary effects (sore throat, nausea, diarrhea), cost and resistance [9]. Hence, discovering new alternative agents is indispensable for the management of this disease.

The use of medicinal plants remains an alternative source of therapies since it contains substances that can be used for therapeutic purposes or that are precursors for the synthesis of useful drugs. Indeed, for millennia, several infections are being treated with medicinal plants [10,11]. Endophytes, microorganisms associated with living plant tissues that produce no apparent indication of their presence in the plant and seem not to cause harm to the host [12,13], are able to produce rich bioactive compounds with a high level of structural diversity, conferring interesting biological activities to them [14-16].

*Cola acuminata* is a slender tree found all over the western cost of Africa. It is used mostly for their economic aspects as well as traditionally and has displayed antimicrobial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Klebsiella pneumoniae* [17]. Endophytic fungi inhabiting *Cola acuminata* have revealed antifungal activities against some multi-resistant *Candida* species [18], although their antibacterial activity remains untapped. As a result, this work design with the aim to evaluate the antibacterial and antioxidant potentials of crude extracts from two endophytic fungi of *Cola acuminata*.

## Materials and Methods

### Materials

The fungal material was made of two endophytic fungi isolated

from the branch (CAB31) and leaf (CAF71) of *Cola acuminata*. Stored at -80°C in 50% glycerol at the antimicrobial and biocontrol agent units. Bacterial strains, including *Staphylococcus aureus* ATCC43300, *Streptococcus pneumoniae* hm145 and isolates, were used for antibacterial assays. The isolates of *Klebsiella pneumoniae* and *Escherichia coli* were obtained from Centre Pasteur of Cameroon. Vero (ATCC CRL 1586) cells were equally obtained from Centre Pasteur of Cameroon, and RAW 264.7 (ATCC #TIB-71) cells were obtained from the Noguchi Memorial Institute for Medical Research, Ghana. The culture media were potato dextrose broth (HIMEDIA) and nutrient broth (LyphiChem). DPPH was from SIGMA.

## Methodology

### Identification of species

The identification of the two species was performed using Fourier transform infrared-attenuated total reflection (FTIR-ATR) spectroscopy by the method formally described [19] with slight modifications.

**Sample preparation:** This consisted of scraping mycelia from solid Leonian's medium after 4 days of growth. The sample was placed onto the ATR crystal (Golden Gate Single Reflection Diamond ATR). The infrared light passed through the crystal and interacted with the sample, which was pressed unto the crystal. Before each fungal sample scan, the device was cleaned with 70% ethanol, and after drying, the background air was taken.

Seven well-characterized fungal strains representing one fungal genus (*Aspergillus* sp., *Trichoderma* sp. and *Fusarium* sp.) obtained from the mycological strain collection of the antimicrobial and biocontrol agent unit (University of Yaoundé 1) were used in this study as a library for comparison purposes.

**Spectral acquisition [19]:** From this, different spectra were obtained showing the specific characteristics of each sample. Spectral acquisition of the strains was performed on a Tensor 27 Fourier transformed infrared spectrophotometer. The environmental conditions were maintained constant (temperature at 25°C and humidity 30%). The spectra were recorded from 4000 to 550 cm<sup>-1</sup> with a resolution of 4 cm<sup>-1</sup> and 260 scans for the sample and background. The scan velocity was 10 kHz, and the interferogram size was 14,220 points. The raw signal obtained was then Fourier transformed to produce a more familiar IR representation of intensity as a function of wave number. Hence, then the name 'FT-IR'. The spectra were acquired and manipulated with Origin Pro software for Windows.

**Chemometry [19]:** The spectra in each region were baseline corrected by applying the rubber band method, normalized separately using the vector normalization method and then offset corrected using Origin Pro software for win cluster analysis. Principal component analysis (PCA) and linear discriminant analysis (LDA) were applied to compare samples and group the spectra with the same degree of similarity. This method calculated the Euclidean distances between all the data sets by using Ward's algorithm method. The merging process was presented in a dendrogram regrouping the different spectra in clusters according to a heterogeneity scale.

### Culture of endophytic fungi and extraction of metabolites

#### Cultivation of endophytic fungi for the production of secondary

**metabolites:** The endophytic extract was prepared according to previous methodology [18,20,21] with few modifications. Briefly, the two species were grown on freshly prepared PDA plates for 4 to 7 days depending on the species. They were introduced in quadruplets into flasks containing 250 ml of nutrient broth (NB) medium and potato dextrose broth (PDB) medium. Nevertheless, only the 1-week culture was carried out for the PDB medium. The culture was then incubated at room temperature for 1, 2, 3 and 4 weeks per species, at room temperature, in the dark and with intermittent shaking. To extract the metabolites, 250 ml of ethyl acetate was added to the culture medium containing the endophytic species (after fermentation), mixed well for 10 minutes and allowed overnight until the two clear immiscible layers were formed. The upper layer of ethyl acetate containing the extract was separated using a separating funnel. The extracts were then concentrated by removing the solvents under reduced pressure at 35-40°C with a rotatory evaporator.

**Determination of minimum inhibitory concentrations (MICs) of extracts against bacterial species:** The MICs of extracts against bacteria were determined as described [22] using the 96-well microtiter plate format.

One hundred microliters (100 µl) of twofold diluted extracts in nutrient broth medium were introduced into the wells of the plate. Thereafter, 100 µl of the bacterial inoculum standardized at 0.5 McFarland were added to each well containing the test substances except the blank column for the sterility control. The concentrations of extracts and the positive control ranged from 3.125 to 100 µg/ml and 0.15625 to 5 µg/ml, respectively. Plates were incubated for 24 hours at 37°C, and turbidity was observed as an indication of growth. The lowest concentration inhibiting the visible growth of bacteria was recorded. Extracts with the best antibacterial activity were selected for the antioxidant and cytotoxic assays.

### In vitro antioxidant and cytotoxicity assays of endophytic fungi extracts

**Scavenging effect on DPPH (2,2-Diphenyl-1-picrylhydrazyl) Radical:** The scavenging effect of the extracts was determined using the protocol previously described [23].

Briefly, 25 µl of extracts prepared at concentrations of 1000, 500, 250, 125 and 62.5 µg/ml was added to 75 µl of methanol solution of DPPH (0.02%) to obtain final volumes of 100 µl and final concentrations of 250, 125, 62.5, 31.25 and 15.625 µg/ml. Vitamin C prepared at an initial concentration of 1 mg/ml was used as a positive control. After 30 minutes of incubation in absolute darkness, the absorbance was read at 517 nm. Each experiment was performed in triplicate, and the percentage of inhibition of endophytic fungal extracts was calculated using the following equation:  $RSA = (A_o - A_s) / A_o \times 100$

where RSA: Radical Scavenging Activity; A<sub>o</sub>: Absorbance of the blank (DPPH + methanol); A<sub>s</sub>: Absorbance of DPPH Radical + endophytic fungi extract.

From %RSA, other parameters, such as the RSA<sub>50</sub>, EC<sub>50</sub>, and ARP, were deduced.

RSA<sub>50</sub> is the concentration of extract at which 50% of the free radicals are scavenged and is obtained from a graph of %RSA as a

function of the logarithmic values of extract concentrations

$EC_{50}$ ; the efficient concentration, defined as the concentration of extract required to scavenge  $\frac{1}{2}$  mole of DPPH, was calculated as follows:

$$EC_{50} = RSA_{50}/[DPPH]$$

ARP; Antiradical power is the inverse of the  $EC_{50}$ . It measures the efficiency of the antiradical; hence, the larger the ARP is, the more efficient the antiradical.

$$ARP = 1/[EC_{50}]$$

**Nitric oxide radical scavenging assay:** The method of Kumaresan et al. [24] with few modifications was employed to determine the nitric oxide radical scavenging activity of the extracts.

Ten microliters (10  $\mu$ l) of the SNP solution was mixed with 25  $\mu$ l of the extracts and vitamin C (positive control) at various concentrations ranging from 6.25 to 50  $\mu$ g/ml. The mixture was incubated at 25°C. After 30 minutes, the solution was mixed with 50  $\mu$ l of Griess' reagent. The mixture was incubated at room temperature for 5 minutes, followed by the measurement of absorbance at 546 nm using a spectrophotometer (Tecan UV-1800).

Each experiment was performed in triplicate, and the radical scavenging activity (RSA) of the extracts was calculated using the following formula:

$$\%RSA = (Ao - As)/Ao \times 100$$

where Ao is the absorbance of the control (SNP + Griess' reagent only) and As is the absorbance of the test samples (SNP + extract + Griess' reagent).

From dose-response curves obtained from different concentrations of the samples, the concentration of sample required to scavenge 50% NO free radicals (50% inhibition concentration,  $IC_{50}$ ) was determined.

**Hydrogen peroxide ( $H_2O_2$ ) scavenging assay:** The method described by Mukhopadhyay et al. [25] was used to determine the hydrogen peroxide ( $H_2O_2$ ) radical scavenging activity of the samples.

To 25  $\mu$ l of extract prepared at different concentrations (500, 250, 125, 62.5, and 31.25  $\mu$ g/ml), 25  $\mu$ l of 5 mM  $H_2O_2$  was added and incubated at room temperature in the dark for 5 minutes. Thereafter, 25  $\mu$ l of  $Fe^{2+}$  (3 mM) was added and incubated for another 5 minutes. After incubation, 75  $\mu$ l of 1 mM 1,10-phenanthroline was added to the sample, homogenized and incubated for 10 minutes at room temperature. Vitamin C served as positive control. Finally, the absorbance was read at 510 nm with a spectrophotometer. The blank solution contained only  $Fe^{2+}$ , distilled water and 1,10-phenanthroline.

Each experiment was performed in triplicate, and the hydrogen peroxide scavenging capacity of the extracts was calculated accordingly.

$$\text{Scavenged } H_2O_2 (\%) = 1 - As/Ao \times 100$$

where Ao is the absorbance of the control ( $Fe^{2+}$  + ortho-phenanthroline) and As is the absorbance of the test ( $Fe^{2+}$  + extract +  $H_2O_2$  + ortho-phenanthroline).

From dose-response curves obtained from different concentrations of the samples, the concentration of sample required to scavenge 50%  $H_2O_2$  (50% inhibition concentration,  $IC_{50}$ ) was determined.

**Ferric ion reducing antioxidant power (FRAP) assay:** The assay was performed according to the method described by Yefrida et al. [26] with slight modifications.

Briefly, 25  $\mu$ l of each test sample was prepared at different concentrations (6.25, 12.5, 25, and 50  $\mu$ g/ml) in the test plates, and 25  $\mu$ l of iron (III) chloride (1.2 mg/ml) was added to the samples. Vitamin C served as positive control. Plates were incubated at room temperature for 15 minutes. After incubation, 50  $\mu$ l of 1,10-phenanthroline (0.02%) was added, and then the absorbance of the mixture was determined at 510 nm through a spectrophotometer. The control contained iron (III) chloride, distilled water and 1,10-phenanthroline.

Each experiment was performed in triplicate, and the reducing capacity of the endophytic fungal extracts was calculated using the following formula:

$$\text{Reducing } Fe^{3+} (\%) = 1 - As/Ao \times 100$$

where Ao is the absorbance of the control ( $Fe^{2+}$  + ortho-phenanthroline) and As is the absorbance of the test ( $Fe^{3+}$  + extract + ortho-phenanthroline).

From dose-response curves obtained from different concentrations of the samples, the concentration of sample required to scavenge 50% of  $Fe^{3+}$  (50% inhibition concentration,  $IC_{50}$ ) was determined.

**Hydroxyl radical antioxidant capacity (HORAC) assay:** Hydroxyl radical scavenging activity was measured by the method of Godlewska-żyłkiewicz et al. [27] with some modifications.

The reaction mixture (100  $\mu$ l) contained 25  $\mu$ l of  $(NH_4)_2 Fe_2SO_4 \cdot 6H_2O$  (5 mM) and 10  $\mu$ l  $H_2O_2$  6 mM, which was left to react to produce OH free radicals in the dark for 45 minutes. Then, 25  $\mu$ l of crude extract at various concentrations (6.25 to 50  $\mu$ g/mL) was added to the wells. The reaction mixture was left to further assess its scavenging activity for 60 minutes at 37°C. Thereafter, 40  $\mu$ l of sodium benzoate 20 mM was added to the plates. The fluorescence was read at 400 nm with excitation at 320 nm. The blank solution contained ferrous ammonium sulfate (25  $\mu$ l, 1 mM),  $H_2O_2$  (10  $\mu$ l), the extracts (25  $\mu$ l) and distilled water (40  $\mu$ l). Gallic acid served as a positive control prepared alongside the crude extracts at similar concentrations to the latter.

Each experiment was performed in triplicate, and the scavenging capacity (RSA) of the endophytic fungal extracts was calculated using the following formula.

$$OH \text{ RSA } (\%) = 1 - As/Ao \times 100$$

where Ao is the absorbance of the control without extract (hydroxyl radical + benzoic acid) and As is the absorbance of the test (hydroxyl radical + extract + benzoic acid).

From dose-response curves obtained from different concentrations of the samples, the concentration of sample required to scavenge 50% OH free radicals (50% inhibition concentration,



IC<sub>50</sub>) was determined.

**In vitro cytotoxicity evaluation of samples:** This test was performed on Vero cells (ATCC CRL 1586) and RAW 264.7 cells (ATCC #TIB-71) using the colorimetric resazurin assay as previously described.

The test was performed in triplicate on 96-well cell culture-treated microplates as previously described. For this, 100 µl of cell suspension was introduced into the wells of 96-well plates to a final charge of 1x10<sup>4</sup> cells/well and incubated overnight at 37°C/5% CO<sub>2</sub>. After this time, the medium was removed and replaced with 96 µl of fresh medium, and 4 µl of each diluted sample was added. Plates were incubated at 37°C in a 5% CO<sub>2</sub> incubator for 48 hours. The positive control contained podophyllotoxin (10 mM) tested at 10 µM, and the negative control wells had cells without. After this time, 10 µl of a solution of resazurin (0.15 mg/ml in PBS) was added to each well and then incubated for 4 hours. Fluorescence of the formed resazurin was measured at excitation and emission wavelengths of 530 nm and 570 nm, respectively, using an InfiniteM200 microtiter plate reader. From the resulting values of optical densities, the percentage of cell viability (CV) was calculated with Microsoft Excel software using the formula:

$$\%CV = (At - Ab) / (At - Ac) \times 100$$

Where,

At = Absorbance of Test, Ab= Absorbance of podophyllotoxin, Ac= Absorbance of negative control (cells).

A dose-response curve of CV against the concentration of the extracts was plotted to determine the 50% cytotoxic concentration (CC<sub>50</sub>).

### Statistical analysis

The spectra were analyzed and manipulated with Origin Pro software for Windows. The data were subjected to One-way Analysis of Variance (ANOVA). Significance differences for multiple comparisons were determined when possible by the Waller-Duncan post hoc test at p≤0.05 using the Statistical Package for the Social Sciences (SPSS, version 16.0) program. Graphical evaluation was performed using Microsoft Excel 2019. RSA50 and IC50 were deducted using Microsoft Excel 2019. GraphPad Prism 5.0 software was used to determine the 50% cytotoxic concentration (CC<sub>50</sub>).

## Results

### Identification of species

FTIR-ATR was used for the identification of CAB31 and CAF71. In the present study, Fourier transform infrared spectroscopy was employed to differentiate between the species. Figure 1 shows the infrared absorption spectra of the two species investigated in this study. The spectra for each fungus were measured from six different isolates.

To differentiate between the two species, we used principal component analysis (PCA) in selected wave number regions. To perform an accurate analysis in this wave number region, all spectra were baseline corrected and normalized. This clustering yielded a good distinction between these two species (Figure 2). Finally, the reference spectra of all isolated species in this study were compared together, and a spectral data library was established.

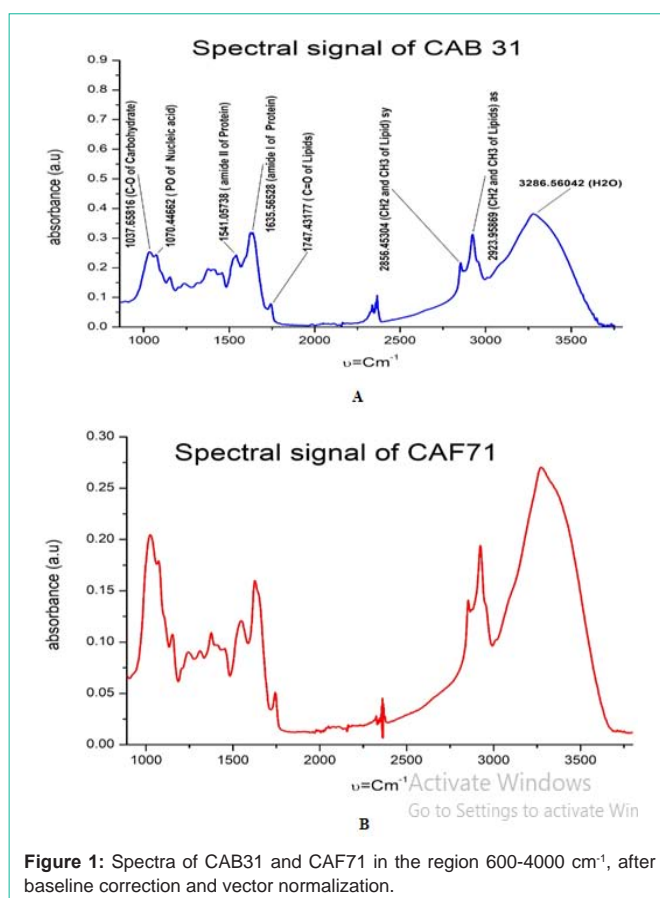


Figure 1: Spectra of CAB31 and CAF71 in the region 600-4000 cm<sup>-1</sup>, after baseline correction and vector normalization.

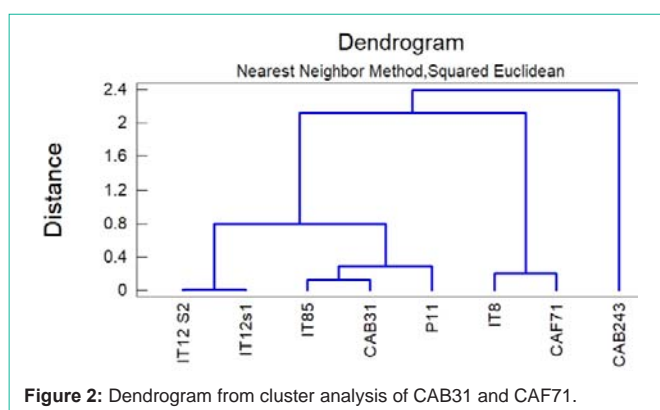


Figure 2: Dendrogram from cluster analysis of CAB31 and CAF71.

Figure 2 shows that CAB31 is in the same cluster as *Trichoderma harzianum* (IT85). In its part, CAF71 belongs to the same cluster as *Trichoderma afroharzianum* (IT8). In that regard, CAB31 and CAF71 were identified as the same species to which they are related.

### In vitro antibacterial activities of endophytic fungi extracts

The screening of extracts was performed at a single concentration of 100 µg/ml, and the results are recorded in the table below.

**Antibacterial screening:** Ten extracts were obtained from *Trichoderma harzianum* and *Trichoderma afroharzianum* from two different culture media and four different incubation times. Table 1 shows that the endophytes produced antibacterial extracts depending on the endophyte species and the culture medium. Globally, the two

**Table 1:** The inhibitory effects of extracts at 100µg/ml on bacterial species.

Sample code	Bacteria Species 100 (µg/ml)				
	SP	SA	KP		EC
CAB31P1	+	+	+		+
CAB31N1	-	-	-		-
CAB31N2	-	-	-		-
CAB31N3	-	-	-		-
CAB31N4	-	-	-		-
CAF71P1	-	-	-		-
CAF71N1	-	-	-		-
CAF71N3	-	-	-		-
CAF71N4	-	-	-		-
CAF71N2	+	+	-		+

+: active; -: not active; CAB31: *Trichoderma harzianum*; CAF71: *Trichoderma afroharzianum*; N: nutrient broth; P: potato dextrose broth; 1: 1-week culture; 2: 2 weeks culture; 3: 3 weeks culture; 4: 4 weeks culture; SP: *Streptococcus pneumoniae*; SA: *Staphylococcus aureus*; KP: *Klebsiella pneumoniae*; EC: *Escherichia coli*.

**Table 2:** Minimum inhibitory concentrations (MICs) of extracts on bacterial species.

Sample	SP	SA	KP	EC
CAB31P1	12.5	12.5	50	100
CAF71N2	50	100	/	100
Gentamicin	0.039	0.078	0.156	/

/: not determined; CAB31P1: *Trichoderma harzianum* 1-week culture in potato dextrose broth; CAF71N2: *Trichoderma afroharzianum* 2-week culture in nutrient broth; SP: *Streptococcus pneumoniae*; SA: *Staphylococcus aureus*; KP: *Klebsiella pneumoniae*; EC: *Escherichia coli*.

extracts were active at 100 µg/ml against the above bacterial strains. *Trichoderma harzianum* P1 was active against all four pathogenic bacteria, while *Trichoderma afroharzianum* N2 was active against all bacteria except *Klebsiella pneumoniae*.

Extracts showing activity on at least three bacterial species were retained for the minimum inhibitory concentration (MIC) determination. These two extracts (CAB31P1 and CAF71N2), with promising activity, were then used for the determination of their MICs.

**Minimum inhibitory concentration (MIC) determination:** The bacterial growth inhibition capacities of the samples were assessed, and their minimum inhibitory concentrations (MICs) are presented in the table below.

From Table 2, MIC values ranged from 12.5 to 100 µg/mL, with *Trichoderma harzianum* P1 showing the best activity against *Streptococcus pneumoniae* and *Staphylococcus aureus* at 25 µg/ml. Generally, the activity of *Trichoderma harzianum* P1 was better than that of *Trichoderma afroharzianum* N2 on the corresponding microorganisms.

From the results, ethyl-acetate extracts of endophytic fungi obtained from the same plant showed different levels of antimicrobial activities. These observed differences in susceptibility could be attributed to the type of isolates, nature and level of the antibacterial agents produced by each endophyte.

**Table 3:** 50% radical scavenging activity (RSA50), 50% efficient concentration (EC<sub>50</sub>) and antiradical power of DPPH radicals.

Sample	RSA <sub>50</sub> (µg/ml)	EC <sub>50</sub> (x 10 <sup>-1</sup> g/mol)	ARP (x10 <sup>3</sup> mol/g)
CAB31P1	687.543 ± 16.943 <sup>a</sup>	13.75 ± 00 <sup>a</sup>	0.072 ± 00 <sup>a</sup>
CAF71N2	642.363 ± 33.657 <sup>a</sup>	12.84 ± 00 <sup>a</sup>	0.077 ± 00 <sup>a</sup>
Vitamin C	5.496 ± 1.046 <sup>b</sup>	0.001 ± 00 <sup>b</sup>	1 ± 00 <sup>b</sup>

Along the line, values carrying the same letter superscripts are not significantly different (P>0.05), Waller Duncan; RSA<sub>50</sub>, 50% Radical scavenging activity; EC<sub>50</sub> = 50% Efficient Concentration; ARP = Antiradical Power; CAB31P1: *Trichoderma harzianum* 1-week culture in Potato dextrose broth; CAF71N2: *Trichoderma afroharzianum* 2-weeks culture in Nutrient Broth.

**Table 4:** 50% inhibition concentration (IC<sub>50</sub>) of samples on NO radicals.

Sample	IC <sub>50</sub> (µg/ml)			
	CAB31P1	CAF71N2	Vitamin C	Gallic Acid
NO	10.456 ± 0.401	12.703 ± 0.401	7.431 ± 0.401	-
FRAP	319.310 ± 5.597	74.756 ± 0.208	52.185 ± 1.709	-
H <sub>2</sub> O <sub>2</sub>	192.160 ± 5.070	253.170 ± 6.934	74.999 ± 13.757	-
OH	12.938 ± 1.716	5.669 ± 1.853	-	23.273 ± 0.264

Along the line, values carrying different letter superscripts are significantly different (P≤0.05), Waller Duncan; IC<sub>50</sub> = 50% inhibition concentration; CAB31P1: *Trichoderma harzianum* 1-week culture in potato dextrose broth; CAF71N2: *Trichoderma afroharzianum* 2-week culture in nutrient broth.

### Antioxidant effects of promising extracts

From dose-response graphs, the 50% radical scavenging activity (RSA<sub>50</sub>) values, 50% efficient concentration (EC<sub>50</sub>) and antiradical power (ARP) were calculated, and the results are recorded in Table 3.

The RSA<sub>50</sub> values of the extracts were 687.543±16.943 µg/ml and 642.363±33.657 µg/ml for *Trichoderma harzianum* P1 and *Trichoderma afroharzianum* N2, respectively. These values were much higher than that of vitamin C (5.496 ± 1.046 µg/ml), highlighting the lower effect of the extracts in comparison to the positive control. From these values, the more efficient the antioxidant capacity of the extracts, the smaller the EC<sub>50</sub> value and the larger the ARP value. The antioxidant screening of the extracts by DPPH assay indicated the presence of antioxidant compounds in the crude extracts, although lower than that of vitamin C, which served as a positive control.

The dose-response graphs obtained were used to determine the 50% inhibitory concentration (IC<sub>50</sub>) of the samples, which are recorded in Table 4.

*Trichoderma harzianum* P1 and *Trichoderma afroharzianum* N2 both revealed nitric oxide chelation effects with IC50 values of 10.456 ± 0.401 µg/ml and 12.703 ± 0.401 µg/ml, respectively. The nitric oxide activity of *Trichoderma harzianum* P1 was significantly higher (p≤0.05) than that of *Trichoderma afroharzianum* N2, both of which were significantly (p≤0.05) lower than that of vitamin C (IC<sub>50</sub> of 4.79 ± 0.10 µg/ml), the positive control. This indicated that the extracts scavenged the nitrite radical to a lower extent than the positive controls.

Regarding the ferric reducing antioxidant power assay, the results in Table 4 clearly show that vitamin C, which served as a positive control, had the greatest reducing capacity with the lowest IC50 value of 52.185 ± 1,709 µg/ml, followed by *Trichoderma afroharzianum* N2 with an IC<sub>50</sub> of 74.756 ± 0.208 µg/ml. The activity of *Trichoderma*

**Table 5:** *In Vitro* Cytotoxicity Evaluation of Raw 264.7 cells and Vero cells samples.

Sample	CC <sub>50</sub> (µg/ml)	
	Raw 264.7 cell	Vero cells
CAB31P1	>100	>100
CAF71N2	>100	>100
Podophyllotoxin	0.141 ± 0.014	0.177 ± 0.058

CC<sub>50</sub> = 50% cytotoxicity concentration; CAB31P1: *Cola acuminata* branch after 1-week of culture in potato dextrose broth; CAF71N2: *Cola acuminata* leaf after 2 weeks of culture in nutrient broth.

*afroharzianum* N2 was significantly greater ( $p \leq 0.05$ ) than that of *Trichoderma harzianum* P1, both of which were significantly lower ( $p \leq 0.05$ ) in activity than vitamin C.

Regarding the hydrogen peroxide assay, the results of their IC<sub>50</sub> values are shown in Table 4. From that table, vitamin C, which served as a positive control, had the greatest scavenging capacity with the least IC<sub>50</sub> value of  $74.999 \pm 13.757$  µg/ml, followed by *Trichoderma afroharzianum* N2 with an IC<sub>50</sub> of  $192.160 \pm 5.070$  µg/ml. The reducing capacities of all the samples were significantly different ( $p \leq 0.05$ ), with *Trichoderma afroharzianum* N2 presenting a better activity than CAB *Trichoderma harzianum* P1 ( $192.160 \pm 5.070$ ).

Table 4 shows that *Trichoderma harzianum* P1 and *Trichoderma afroharzianum* N2 possessed significantly better ( $p \leq 0.05$ ) hydroxyl radical scavenging activities with IC<sub>50</sub> values of  $12.938 \pm 1.716$  µg/ml and  $5.669 \pm 1.853$  µg/ml, respectively, than gallic acid ( $23.273 \pm 0.264$ ), which served as a positive control.

Overall, although less than the reference in most cases, the antioxidant activity of *Trichoderma afroharzianum* N2 was significantly higher than that of *Trichoderma harzianum* P1 on the respective radicals.

### **In vitro cytotoxicity evaluation of samples**

Table 5 below shows the 50% cytotoxicity concentration of samples on two cell lines.

According to the National Institute of Cancer, an extract is considered cytotoxic when it shows a CC<sub>50</sub> value less than 20 µg/ml. The table above presents the CC<sub>50</sub> values of the active samples, which are all greater than 100 µg/mL. All samples were therefore not cytotoxic (CC<sub>50</sub> values > 20 µg/ml) to either Raw or Vero cells.

## **Discussion**

This work was designed with the aim of obtaining extracts with antibacterial and antioxidant properties from endophytic fungi inhabiting *Cola acuminata*. To achieve this goal, two endophytic fungi isolated from *Cola acuminata* were used. The identification of these strains was performed by FTIR-ATR spectroscopy. FTIR-ATR spectroscopy is an attractive technique for the detection and identification of pathogens. This enabled us to learn about the spectral relations between the various isolates, which in turn helped us track the propagation of the fungus and the origin of a particular isolate [19]. The present study successfully identified two *Trichoderma harzianum* and *Trichoderma afroharzianum* isolates from *Cola acuminata*. This goes in the straight line of what was done recently [19-21] based on the suggestion done previously [22-25]. The identified endophytic fungi *T. harzianum* and *T. afroharzianum* were then cultured with

two different culture media (PDB and NB) and at different culture durations and were subjected to ethyl acetate extraction.

Antibacterial screening revealed that of the 10 extracts obtained, CAB31P1 (*Trichoderma harzianum*) showed activity against all four pathogenic bacterial strains, while CAF71N2 (*Trichoderma afroharzianum*) showed activity against three bacterial strains. No extract cultivated in NB medium for 1 week exhibited any activity; hence, this could be because NB medium below 1 week is not appropriate for the production of active principles against these pathogens. This is in accordance with what has been reported elsewhere. In fact, Vrabl et al. reported that the biomass of *Trichoderma harzianum* is correlated with the chemical composition of the culture medium [26]. It is known that secondary metabolite production is correlated with the chemical composition of the medium. It has been reported that when nutrients are going down, endophytes start producing active compounds against other microorganisms that can compete with them for the substrate present in the milieu [15,27,28]. Likewise, the two-week culture in NB showed activity, which could be because the fungi produced more active metabolites with adaptation during its growth phase [26]. The antibacterial activity of these two crude ethyl acetate extracts, CAB31P1 and CAF71N2, which exhibited inhibitory activities at 100 µg/mL on at least three bacterial strains, was then evaluated by determining their MICs. Hence, both extracts displayed significant activity on all bacteria, but CAB31P1 revealed the lowest MICs of 12.5 µg/mL on *Streptococcus pneumoniae* and *Staphylococcus aureus*. These results are in agreement with previous studies [16] although these are the first antibacterial results of endophytic fungi isolated from *Cola acuminata*.

Having unraveled the possible antibacterial activity of the extracts and characterized the relationship between their concentrations and the culture media from which they were extracted, their antioxidant potentials were equally evaluated to determine whether, in addition to their antibacterial properties, they could fight against possible oxidative stress caused by pathogenic invasion. First, in regard to Table 3, the extracts had comparable DPPH scavenging activities, which were significantly lower than that of vitamin C. CAB31P1 and CAF71N2 both revealed anti-nitric oxide activities with IC<sub>50</sub> values of  $10.456 \pm 0.401$  µg/ml and  $12.703 \pm 0.401$  µg/ml, respectively, which were significantly closer to that of the positive control (Table 4). Similarly, CAF71N2 scavenged hydrogen peroxide with an IC<sub>50</sub> of  $192.160 \pm 5.070$  µg/ml (Table 4). To the best of our knowledge, we did not find authors who discussed the nitric oxide- and peroxide-scavenging effects of endophytic extracts. CAB31P1 and CAF71N2 displayed better hydroxyl radical scavenging activities with IC<sub>50</sub> values of  $12.938 \pm 1.716$  µg/ml and  $5.669 \pm 1.853$  µg/ml, respectively (Table 4), than gallic acid. Regarding the ferric reducing capacity of the extracts, vitamin C, which served as a positive control, had the greatest scavenging capacity with the lowest IC<sub>50</sub> value of  $52.185 \pm 1.709$  µg/ml, which was closer to that of CAF71N2 with an IC<sub>50</sub> of  $74.756 \pm 0.208$  µg/ml (Table 4). To the best of our knowledge, there is no report on the antioxidant effect of the *Trichoderma* sp extract in general and, more specifically, on the extract isolated from *Cola acuminata* from Cameroon. This result cannot be compared with other endophytes, as they have different synthetic abilities, and as observed in the present report, two closely related endophyte species do not have the same secondary metabolite synthetic abilities.



This goes in the same line with what is established in the literature [13,18,29-31].

However, in the search for new therapeutic solutions to pathogens, the greatest worry remains the desire to find these solutions without them having undesirable side effects, such as the frequent cytotoxicity of isolated, semisynthetic or synthetic compounds. Hence, regardless of the therapeutic potential of a sample, it is necessary to evaluate its cytotoxicity. The cytotoxicity of the samples was evaluated on Vero cells and Raw 246.7 cells. Their CC50 were all greater than 100 µg/mL. This finding is in line with the report from Damour et al., which showed that pretrichodermamide A and nafuredin isolated from *trichoderma* sp harbored by *Cola nitida* did not have cytotoxic effects on the mouse lymphoma cell line L5178Y [29].

## Conclusion

In the present study, CAF71 and CAB31 were *Trichoderma harzianum* and *Trichoderma afroharzianum*, respectively. The culture of *Trichoderma harzianum* in potato dextrose broth for 1 week and *Trichoderma afroharzianum* in nutrient broth for 2 weeks displayed the best antibacterial and antioxidant effects with no cytotoxic effect on the cell lines tested. To the best of our knowledge, this is the first report on the antibacterial and antioxidant activities of endophytic fungal extracts from *Cola acuminata*.

## Author Contribution Statement

J.B.H. F, E.R.M and L.V.Y. performed the experiments, J.B.H.F., P.K.L, E.R.M, A.V.M., L.V.Y., contributed samples/materials/analyzed the data and drafted the article. P, M.R., A.V.M., conceived and designed the experiments. All the authors critically revised the work.

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