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HPLC Fingerprinting and *In vitro* Antimycobacterial Activity of the Roots of *Cissampelos owariensis* and *Cissampelos mucronata*

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ABSTRACT

Two Nigerian medicinal plants *Cissampelos owariensis* and *Cissampelos mucronata* (Menispermaceae) are commonly used in traditional medicine for the management of tuberculosis-related symptoms. The rationale behind this study is based on the fact that the two plants possess similar appearances thus often mistaken for each other, and both have also been reported for antimycobacterial activity. Hence, the objective of this study was to profile the chemical constituents of the two plants, establish their respective chromatographic fingerprints as an identity marker, and compare their bioactivities against two *Mycobacterium* species. Aqueous methanol extract of the roots of both plants was screened for their secondary metabolite contents and were also evaluated for their action against *Bacillus Camille Guerin* and *Mycobacterium smegmatis*. Alkaloids, flavonoids, steroids and terpenes were present in both plant extracts. *In-vitro* antimycobacterial assay showed that the extracts of *C. owariensis* and *C. mucronata* inhibited the growth of *M. smegmatis* at 3.13 mg/mL and 6.25 mg/mL, respectively and was bactericidal at 6.25 mg/mL and 12.5 mg/mL, respectively. Against BCG, the extract of *C. owariensis* displayed inhibitory and bactericidal properties at 0.39 mg/mL and 0.78 mg/mL, respectively, and *C. mucronata* at 3.13 mg/mL and 6.25 mg/mL, respectively. This indicates that both strains of the *Mycobacterium* were more susceptible to *C. owariensis* than *C. mucronata in-vitro*. The HPLC fingerprint results were non-identical for the two plant extracts, and comparison with their alkaloid fraction chromatograph revealed that the crude extracts consisted largely of alkaloids. This study has established distinguishable chromatographic profiles between the two species.

Keywords: *Cissampelos owariensis*, *Cissampelos mucronata*, Antimycobacterial, HPLC, Quality control.

Introduction

Tuberculosis (TB), a chronic bacterial infection caused by *Mycobacterium tuberculosis*, is reported to rank as the thirteenth leading cause of death worldwide and the second leading cause of death from a single infectious agent after COVID-19.[1] Multi (MDR), extensively (XDR), extremely (XXDR), and total (TDR) drug-resistant strains of the bacterium have emerged in the last two decades as a threat to global public health emphasizing the urgent need to develop new tuberculosis (TB) therapeutic strategies. The global economic cost implication of drug-resistant (TB) currently stands at 16.7 trillion US dollars.[2] There has been considerable interest in developing potential drugs from medicinal plants for treating tuberculosis. Ibekwe *et al* carried out an ethnobotanical study on traditional recipes composed mainly of plants that were used in Nigeria for the management of tuberculosis and related symptoms. Their findings revealed that *Bacillus Camille Guerin* (BCG) was susceptible to 69% of the recipes with activities at ≤ 500 µg/mL–2500 µg/mL.[3]

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Nigeria is endowed with a rich biodiversity of medicinal flora and a large percentage of the population domiciled particularly in the rural areas depends on the traditional medicine system for their primary health care. *Cissampelos owariensis* and *Cissampelos mucronata* both belonging to the family Menispermaceae are important medicinal plants, much widely spread over Africa and grow in a variety of habitats. They both have close physical similarities and more so, they are used for similar therapeutic purposes making it often difficult to distinguish between the species.[4] Thus the roots of both plants are used to treat chronic cough and the anti-tubercular potentials of both plants have also been reported.[5,6] An infusion of the bitter rhizome, leaves or stems of *C. owariensis* P. Beauvais is used to cure gastrointestinal complaints such as diarrhoea, dysentery, colic, and intestinal worms, and in the treatment of urogenital ailments such as menstrual problems, venereal diseases and infertility.[7,8] The leaves and rhizomes or their ash are widely used in various forms to treat abscesses, ulcers and scabies.[9] In Nigeria, the plant is reported to be used in the treatment of lung diseases.[10] Two sesquiterpenes and other chemical constituents have been isolated from the roots of this plant species.[6,11] The root extracts were reported to have displayed antimycobacterial activity against three different strains of *M. tuberculosis*.[6] The rhizome of *C. mucronata* A. Rich, on the other hand, is used in West African countries for the treatment of catarrh, tonsillitis, cough, oedema, and lung infections, and similar to *C. owariensis*, also used to prepare arrow poisons in Nigeria.[4] In the East of Africa, the rhizome decoction is administered for the treatment of fever caused by malaria or jaundice while the sap is formulated as ear drops to treat ear aches.[4] Furthermore, in Southern parts of Africa, the

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rhizome is used as an ingredient in the treatment of cough, headache, neck pain, lumbago, bilharzia, and wound healing.[12] All the plant parts of *C. mucronata* are rich in alkaloids; the classes of alkaloids detected include bisbenzylisoquinoline, aporphine, proaporphine and morphinane alkaloids.[4] The root extracts of *C. mucronata* were reported to show anti-plasmodial properties, with activity against *Trypanosoma cruzi* and *Trypanosoma rhodensiense*, chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum* strains.[13-15] Other pharmacological properties of the root extracts include sedative effect, antimicrobial, larvicidal, molluscicidal, anti-ulcer, uterine relaxant, and anti-tuberculosis activities.[5,16-21] BCG is a slow-growing, non-virulent strain closely related in terms of genetic composition and drug susceptibility profiles to *M. tuberculosis* $H_{37}R_v$, which is the representative virulent strain of the organism. *M. smegmatis* is a rapid-growing non-virulent, saprophytic, surrogate *Mycobacterium* specie. Many researchers prefer to work with these strains because of the difficulty and practicability in meeting the biosafety guidelines for investigating *M. tuberculosis* $H_{37}R_v$ which demand the use of laminar-flow hoods and level 3 facility equipment.[22] The rationale behind this study is that closely-related plant species possessing similar appearances can often be mistaken for each other, and in this case, both species of *Cissampelos* have been reported for antimycobacterial activity and used traditionally for almost the same ailments. Hence, this study seeks to investigate both plants, profile their chemical constituents, establish their chromatographic fingerprints as a discriminating quality control marker, and compare their anti-tuberculosis efficacy.

Materials and Methods

General experimental procedures
BCG and *M. smegmatis* ATCC 607 were cultured in the Diagnostic laboratories at the National Institute for Pharmaceutical Research and Development (NIPRD), Nigeria with Middlebrook 7H9 broth obtained from Difco, Detroit, Michigan. The organisms were reconstituted in Middlebrook 7H9 broth, supplemented with 0.2% (v/v) glycerol, 1.0 g of Casitone per liter, 10% (v/v), OADC (oleic acid, albumin, dextrose, catalase). All the reagents used for the HPLC fingerprinting were of HPLC grades, while those used for phytochemical screening were of analytical grades. HPLC analysis was performed on an Agilent 1100 series equipped with a quadruple pump, degasser and a variable wavelength detector. Sample injection was carried out manually via a 20 µL loop.

Plant collection and identification
The roots of *C. owariensis* were obtained from Suleja, Niger State, Nigeria while those of *C. mucronata* were collected from Abuja, Nigeria in February, 2020. Specimens of both plants with voucher numbers NIPRD/H/7229 and NIPRD/H/7221, respectively were identified at the Herbarium Unit of NIPRD, Abuja. The plant materials were air-dried, pulverized and stored at room temperature.

Preparation of crude extracts
Three hundred grams (300 g) of the air-dried roots of *C. owariensis* and *C. mucronata* were individually macerated in 3 L 90% aqueous methanol for 48 hours. Crude extracts coded COE and CME, respectively were obtained after recovery of the organic solvent using a rotary evaporator and were further dried over a water bath at 100 °C to remove the residual water.

Extraction of crude alkaloid portions
The crude alkaloid portion present in COE and CME was extracted using the method of Yu si *et al*.[23] Ten grams (10 g) of COE was dissolved in distilled water and acidified with a sufficient volume of 2M HCl to attain a pH of 2. The solution was then partitioned with an equal volume of ethyl acetate to remove the non-alkaloids. Thereafter, the aqueous portion was basified with 10 M NaOH to a pH of 10 to precipitate the alkaloids, which were then partitioned with dichloromethane (1:1). The separated dichloromethane layer was concentrated with a rotary evaporator to obtain the alkaloid-rich fractions of aqueous methanol extract of *C. owariensis* (coded COE-A). Thin layer chromatography (TLC) analysis on the alkaloid rich fraction was performed in a solvent system of chloroform and methanol (4: 1) using Dragendorff's reagent as a detector and an orange colouration of the separated spots confirmed the presence of alkaloids. The same procedure was repeated for CME and the crude alkaloid portion was coded CME-A.

Qualitative screening of extracts
COE and CME were qualitatively assessed for the presence or absence of secondary metabolites according to the standard procedures described by Khalid *et al*.[24]

HPLC Fingerprint of the crude extract and the crude alkaloid extract.
HPLC sample preparation was carried out by dissolving 5 mg of COE, COE-A, CME, and CME-A in 10 mL of absolute ethanol to achieve a concentration equivalent to 0.5 mg/mL of each sample. Each resulting solution was filtered using a 0.45 μm membrane filter. Thereafter, 20 μL of each filtrate was injected into the HPLC for fingerprint analysis. Each injection was carried out in duplicates to ensure reproducibility. A suitable HPLC-UV method of separation (Purospher® STAR RP-18 endcapped column, 4.6 mm × 250 mm, 3 μm, Acetonitrile and distilled water as the mobile phase in an isocratic elution mode with the appropriate ratio determined per extract) was used for the fingerprint analysis after some method development procedures such as sample solvent optimization, mobile phase optimization and choice of HPLC column. The detection wavelength was set at 254 nm, the column temperature was ambient, and the flow rate was set at 0.7 mL/min.

Determination of minimum inhibitory concentration
The broth-microdilution method as described by Caviedes *et al* and Ncube *et al* was employed in this study.[25,26] BCG and *M. smegmatis* were the test organisms. Two grams (2 g) of COE and CME each were dissolved in 1 mL of dimethyl-sulphoxide (DMSO) using a vortex mixer and 9 mL of sterile distilled water was added to give a concentration of 200 mg/mL. A further 1:2 dilution was made to obtain 100 mg/mL as the starting working extract solution. 50 µL of sterile Middlebrook medium was dispensed into 96 well micro-plates. 50 µL of extract solution was dispensed each into the labeled well first well (1:2) followed by serially transferring 50 µL of the mixture to the 2^{nd} well and 3^{rd} well and so on to the 9^{th} well. The 10^{th}, 11^{th} and 12^{th} wells were extract sterility, media and organisms viability controls, respectively. The above procedure was carried out in triplicates. 0.02 g of rifampicin powder was dissolved in 10 mL sterile distilled water to afford a concentration of 0.002 mg/mL. 50 µL of rifampicin solution was dispensed as a positive control at the same concentration of 0.002 mg/mL in all the wells. The 5-day cultures of BCG and *M. smegmatis* were adjusted to an optical density (OD) of 0.5 McFarland turbidity standard. 50 µL each of BCG and *M. smegmatis* was dispensed into the labeled 96 microplates and incubated at 37 °C for 7 days. The tetrazolium solution (dye) was added to the 96 wells for the monitoring of growth by the colour change in the inoculated wells. The highest dilution that inhibited the growth of the BCG or *M. smegmatis* was taken as the minimum inhibitory concentration (MIC) of each test sample.

Determination of minimum bactericidal concentration
The method of Baron *et al* was adopted.[27] One hundred microliters (100 µl) each of inoculum from the wells that showed no colour change was transferred and spread on the freshly prepared sterile Middlebrook 7H10 agar plates and further incubated for 7 days at 37°C. The plates were compared with initial colonies of BCG and *M. smegmatis* and counts that were found to be less than 0.1% indicated the minimum bactericidal concentration (MBC) of extracts.

Results and Discussion

Aqueous methanol extraction of the roots of *C. owariensis* and *C. mucronata* yielded 15.1% and 18.6% of COE and CME, respectively while the alkaloid extraction of COE and CME afforded 1.3% and 1.7% of COE-A and CME-A, respectively. Phytochemical screening

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of COE and CME revealed the presence of alkaloids, flavonoids, steroids, and terpenes (Table 1). The component peaks for the chromatographic fingerprints of COE and CME showed relatively acceptable resolutions. The fingerprint chromatogram for COE in Figure 1 was characterized by eight notable peaks at the following distinguishable retention times; 8.23 mins (Peak 1), 8.59 mins (Peak 2), 10.04 mins (Peak 3), 10.95 mins (Peak 4), 14.10 mins (Peak 5), 24.19 mins (Peak 6), 31.14 mins (Peak 7), 32.91 mins (Peak 8), with CH_3CN and distilled H_2O (57:43) as mobile phase. In Fig. 2, the fingerprint chromatogram for CME extract was characterized by five distinct peaks at the following retention times; 5.13 mins (Peak 1), 6.06 mins (Peak 2), 7.46 mins (Peak 3), 11.39 mins (Peak 4), and 13.67 mins (Peak 5), with CH_3CN and distilled H_2O (50:50) as mobile phase. Furthermore, the chromatographic fingerprints showed sets of reproducible detectable component peaks, in which the peak-to-peak distribution patterns were stable and consistent. The HPLC fingerprint results from this study were non-identical for the two plant extracts, which confers a measure of distinction between the two species. HPLC fingerprint analysis has proven to be a veritable quality control tool to distinguish between extracts of plant species of the same genus.[28,29] The established fingerprint method for COE and CME is convenient and feasible as a tool for plant species authentication and quality assessment of the plant extracts.[30]

Table 1: Phytochemical screening for metabolites of *C. owariensis* and *C. mucronata*

Test	*C. owariensis*	*C. mucronata*
Tannins	-	+
Saponins	-	-
Flavonoids	+	+
Anthraquinones	-	-
Steroids	+	+
Terpenes	+	+
Alkaloids	+	+
Cardiac glycosides	-	+

Key: + = positive, - = negative

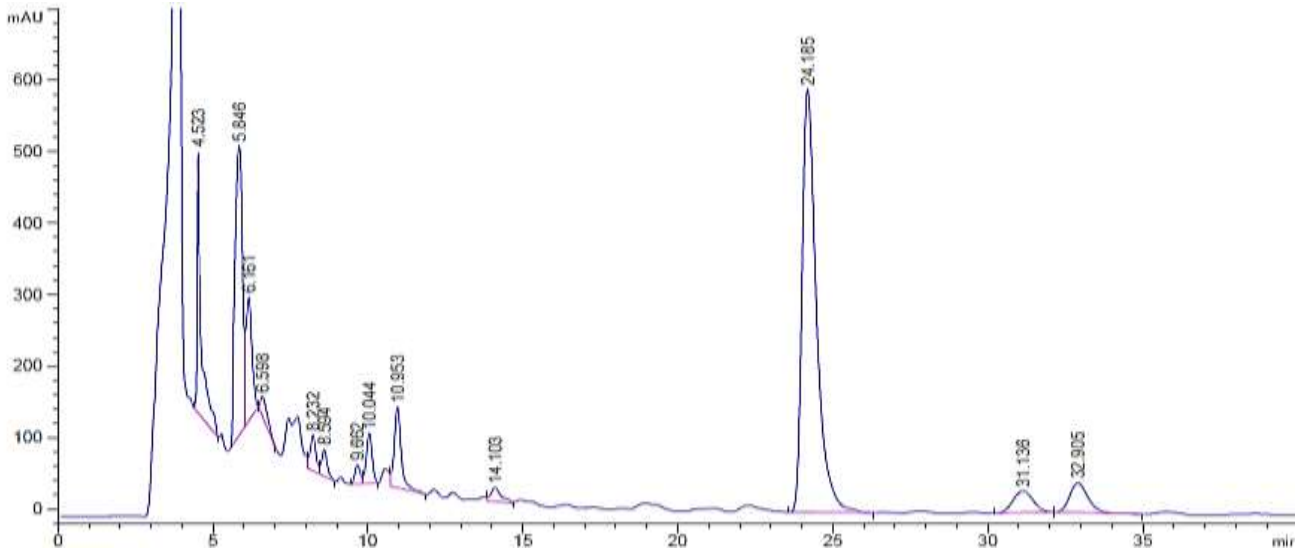

Figure 1: Chromatogram showing the fingerprint separation of COE with a HPLC-UV method conditions of 57% CH_3CN, 254 nm, and 0.7 mL/min in an isocratic elution

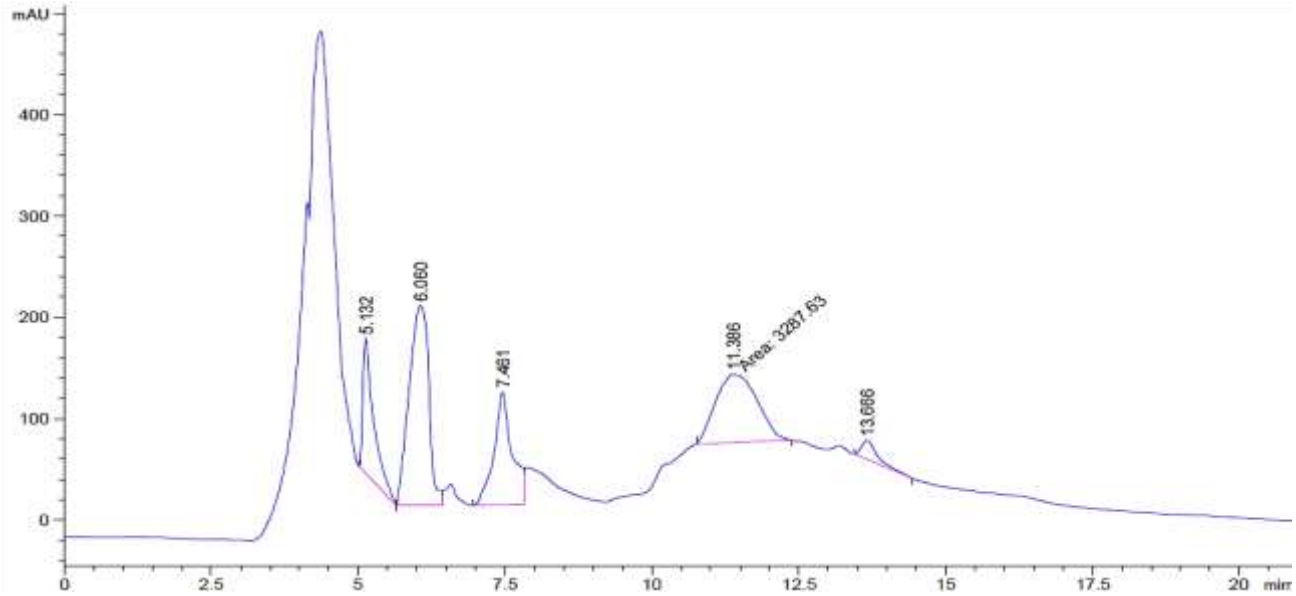

Figure 2: Chromatogram showing the fingerprint separation of CME with a HPLC-UV method conditions of 50% CH_3CN, 254 nm, and 0.7 mL/min in an isocratic elution.

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Furthermore, through the utilization of the peculiar retention times of the characteristic peaks in the fingerprint chromatogram for each analyzed sample, extracts of *C. owariensis* and *C. mucronata* can be authenticated by using the applied HPLC fingerprint method. During this study, the HPLC fingerprint for the alkaloid fractions from both plant species; COE-A and CME-A, were also analyzed. The HPLC fingerprint analysis of COE-A resulted in 9 notable well-separated peaks at 8.66 mins (Peak 1), 9.70 mins (Peak 2), 10.11 mins (Peak 3), 11.03 mins (Peak 4), 14.17 mins (Peak 5), 21.34 mins (Peak 6), 24.37 mins (Peak 7), 31.37 mins (Peak 8), 33.13 mins (Peak 9), with CH_3CN and distilled H_2O (57:43) as mobile phase (Figure 3), while that of CME-A yielded the following distinguishable peaks at 5.27 mins (Peak 1), 5.99 mins (Peak 2), 6.42 mins (Peak 3), 6.59 mins (Peak 4), 7.51 mins (Peak 5), 9.52 mins (Peak 6), 11.67 mins (Peak 7), 11.82 mins (Peak 8), 13.18 mins (Peak 9), 13.73 (Peak 10) with CH_3CN and distilled H_2O (50:50) as mobile phase (Figure 4). The chromatographs for both COE and COE-A were superimposed and compared, it was observed that a good number of the peaks in the COE chromatogram thus matched with those of the alkaloid portion COE-A, as shown in Figure 5. The same observation was noted between CME and the alkaloid portion CME-A (Figure 6), and these observations are in sync with the literature report that the main metabolites found in this genus are alkaloids.[31-34] This is particularly true with regards to *C. mucronata*, but for *C. owariensis* constituents, our study reports this observation for the first time via the demonstration of a simple chromatographic fingerprint comparison between the crude methanol extract and the crude alkaloid fraction. The results of the phytochemical screening concerning the fingerprint analysis confirmed the presence of alkaloids while revealing the presence of other secondary metabolites. Tables 2 and 3 represent the anti-TB activity of COE and CME against *M. smegmatis* and BCG at different concentrations in comparison with rifampicin. COE at 3.13 mg/mL inhibited the growth of *M. smegmatis*, and had a bactericidal action at 6.25 mg/mL. The extract exhibited a higher activity against BCG at the concentration of 0.39 mg/mL (MIC) and 0.78 mg/mL (MBC). CME also demonstrated an anti-TB activity against *M. smegmatis* at 6.25 mg/mL (MIC) and was bactericidal at 12.5 mg/mL (MBC). However, the MIC for CME against BCG was lower at 3.13 mg/mL while the MBC was at 6.25 mg/mL. The results suggest that BCG was more susceptible to COE and CME than *M. smegmatis*. In a previous study, the root extracts of *C. owariensis* were evaluated against three strains of *Mycobacteria*; a resistant strain MDR048, a mixed strain ZMC128 and a susceptible strain ZMC128. The strains were all susceptible to the extracts with a MIC of 3.32 mg/mL.[6] In a similar study in which nine Nigerian plants were assessed against BCG, the roots of *C. mucronata* showed activity at 5 mg/mL.[5] The findings are comparable with the results of the present study which further provide support to the anti-tuberculosis potentials of the plants.

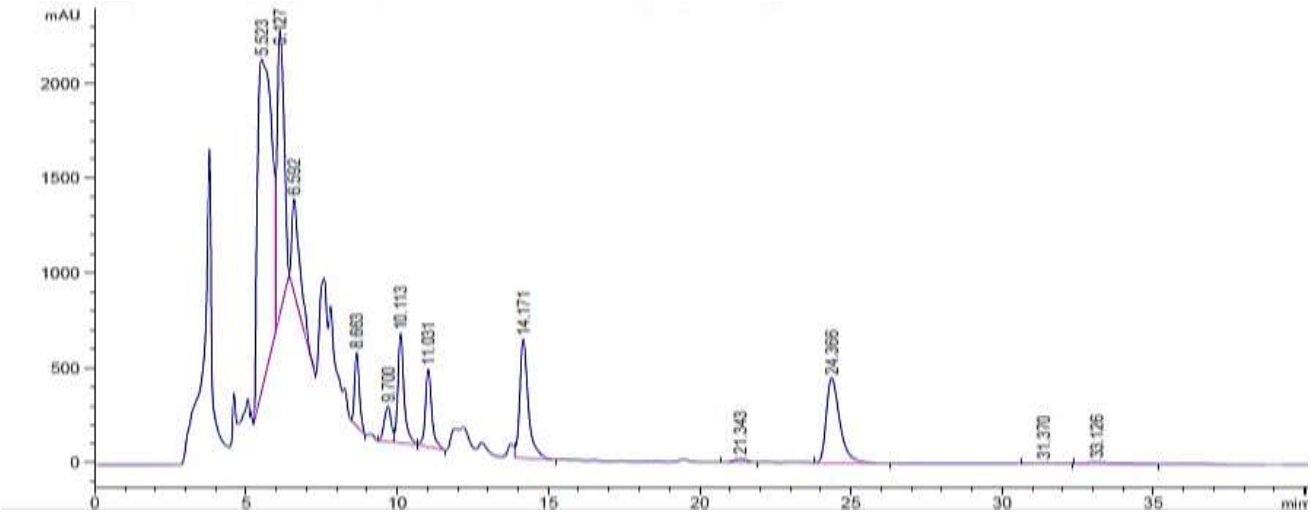

Figure 3: Chromatogram showing the chromatographic fingerprint of the crude alkaloid extract from COE-A with a HPLC-UV method conditions of 57% CH_3CN, 254 nm, and 0.7 mL/min in an isocratic elution.

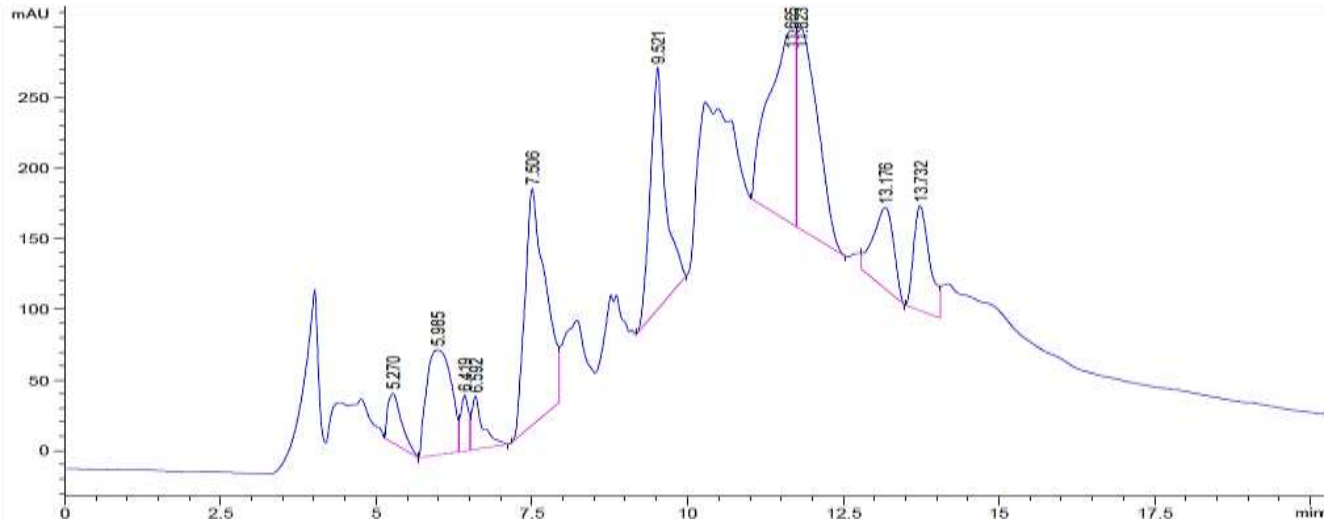

Figure 4: Chromatogram showing the chromatographic fingerprint of CME-A with a HPLC-UV method condition of 50% CH_3CN, 254 nm, and 0.7 mL/min in an isocratic elution.

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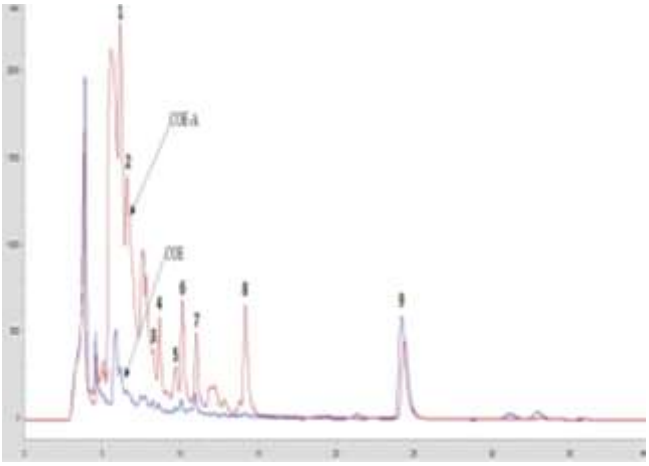

Figure 5: Superimposed Chromatograms of both COE and COE-A showing similarity of constituents.

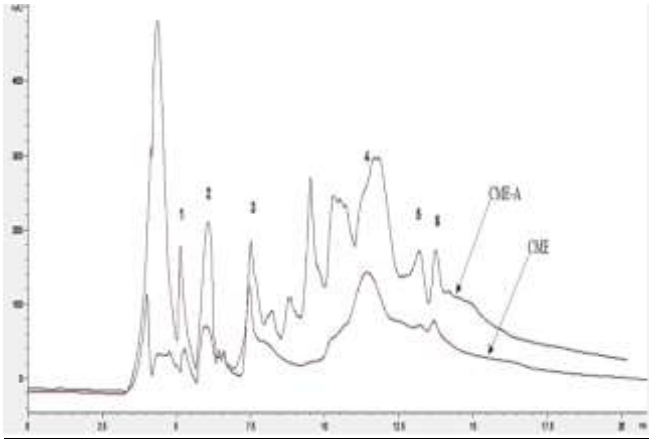

Figure 6: Superimposed Chromatograms of both CME and CME-A showing similarity of constituents.

Table 2: MIC AND MCB of *C. owariensis* and *C. mucronata against M. smegmatis*

Test Sample	Conc (mg/mL)	Well									MIC	MBC
		1	2	3	4	5	6	7	8	9		
COE	100	-	-	-	-	-	+	+	+	+	3.13	6.25
CME	100	-	-	-	-		+	+	+	+	6.25	12.5
Rif	0.002	-	-	-	-	-	-	-	-	-	0.001	

Key: (-) No growth, (+) Growth, (MIC) minimum inhibitory concentration, (MBC) minimum bactericidal concentration, (Rif) rifampicin

Table 3: MIC AND MCB of *C. owariensis* and *C. mucronata against* BCG

Test Sample	Conc (mg/mL)	Well									MIC	MBC
		1	2	3	4	5	6	7	8	9		
COE	100	-	-	-	-	-	-	-	-	+	0.39	0.78
CME	100	-	-	-	-	-	+	+	+	+	3.13	6.25
Rif	0.002	-	-	-	-	-	-	-	-	-	0.001	

Key: (-) No growth, (+) Growth, (MIC) minimum inhibitory concentration, (MBC) minimum bactericidal concentration, (Rif) rifampicin

Conclusion

HPLC analysis can serve as quality control in the identification and differentiation of aqueous methanol extracts of *C. mucronata* and *C. owariensis*. The study has demonstrated that aqueous methanol extracts of *C. mucronata* and *C. owariensis* possess moderate *in vitro* antimycobacterial activity against *M. smegmatis* and BCG in comparison to rifampicin. The study justified the use of these plants in the management of tuberculosis and related symptoms by folks and traditional medicinal practitioners.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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