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Original Article

Antioxidant, antibacterial, antifungal activities and gas chromatographic fingerprint of fractions from the root bark of *Afzelia africana*

Bright Yaw Vigbedor[1], Clement Osei Akoto[2], Ralph Kwakye[1], Jonathan Osei-Owusu[3], David Neglo[1], Pius Kwashie[1]

[1]*Department of Basic Sciences, School of Basic and Biomedical Sciences, University of Health and Allied Sciences, PMB 31, Ho, Ghana;* [2]*Department of Chemistry, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana;* [3]*Department of Biological, Physical and Mathematical Sciences, University of Environment and Sustainable Development, PMB, Somanya, Ghana*

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Abstract: Background: *Afzelia africana* is a tropical plant with numerous ethno-medicinal benefits. The plant has been used for the treatment of pain, hernia, fever, malaria, inflammation and microbial infections. Objectives: To perform bioassay-guided fractionation, antioxidant and antimicrobial activities of the bark of *Afzelia africana*. Methods: Column chromatography fractionation, antioxidant activity (% (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and 1,1-diphenyl picrylhydrazyl (DPPH) scavenging activity))), antimicrobial activity (microbroth dilution: Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), MBC/MIC ratio), and synergistic activities (Checkerboard assay: Fraction Inhibitory Concentration Index (FICI)). Results: Bioassay-guided fractionation of *A. africana* produced four fractions that displayed promising free radical scavenging activities in the ABTS (54-93)% and the DPPH (35-76)% assays in the ranking order of F1(93-54)>F4(81-58)>F2(74-58)>F3(72-55) and F3(77-42)>F1(64-46)>F4(55-44)>F2(47-35) respectively at a concentration range of 1.0-0.01 mg/mL. The fraction F1 (MBC: 2.5-5.0 mg/mL) and F4 (MBC: 1.25-10.0 mg/mL) exhibited broad spectrum of superior bactericidal effects than F2 (MBC≥100.0 mg/mL) and F3 (MBC: 12.5-100.0 mg/mL) against *Staphylococcus mutans*, *Staphylococcus aureus*, *Escherichia coli*, fluconazole-resistant *Candida albicans*, methicillin-resistant *S. aureus*, *Bacillus subtilis*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa, Salmonella typhi,* and *Candida albicans* (standard strain). The two most active fractions (F1 and F4) reported synergistic effects (FICI≤0.5) against *S. typhi* whilst the F4 reported additional synergism against *E. coli*, *K. pneumonia,* and *S. typhi* when combined with ciprofloxacin. Furthermore, the two fractions reported synergistic effects against *Escherichia coli*, *Klebsiella pneumonia*, *Salmonella typhi,* and *Pseudomonas aeruginosa* when combined with tetracycline whilst F1 reported antifungal synergism against fluconazole resistant *Candida albicans* when combined with fluconazole and ketoconazole. Conclusion: The study has confirmed the antioxidant, antimicrobial and synergistic uses of *A. africana* for the treatment of both infectious and non-infectious disease.

Keywords: *Afzelia africana*, antimicrobial, minimum bactericidal concentration (MBC), antioxidant, bioassay-guided fractionation, fractional inhibitory concentration index (FICI)

Introduction

The continuous quest for treatment interventions against microbial infections has derived their route from both natural and synthetic compounds [1, 2]. The World Health Organization through policy formulations has promoted and enhanced the patronage of plant medicines in the midst of the unending phenomenon of drug resistance [3]. Research into natural products has provided route to the discovery of potent antimicrobial compounds with wide structural varieties and complexities. Moreover, the attention has equally drifted towards the search and standardization of antimicrobial compounds from plants [4].

Bioassay-guided fractionation is a useful analytical technique for the simultaneous screening of bioactive samples and the determination of biological activities through the application of different solvent systems and stationary phases [5]. The technique has provided useful information about secondary metabolites from plants and their biological activities that were not originally present in their crude extracts [5, 6]. Furthermore, the technique has provided rich information on the efficacy of combination therapy of different natural antimicrobial and the positive effects of their synergistic effects on microbial lethality [6].

Currently, advanced chromatographic and spectroscopic techniques have helped to obtain total phytochemical content and fingerprint of herbal medicines [7, 8]. Specifically, Gas Chromatography (GC) is an analytical technique based on the principle of passing vaporized samples through a porous stationary phase and measuring parameters such as the retention time, peaks heights, areas and their percentages [9]. The technique has been utilized as a standardization and quality control tool to evaluate the clinical efficacy of herbal formulations by providing information on the composition of bioactive compounds in plant formulations [9].

It is estimated that, about 80% of the developing world depend on plants for their medical needs [10, 11]. Consequently, preliminary information on the medicinal potentials of plants over the years have been solicited through ethnobotanical surveys [12]. Additionally, the total number and quantity of bioactive compounds in plants may vary with both the locality and the season in which they are collected [5]. *Afzelia africana* is a plant species with numerous ethnobotanical and laboratory-confirmed biological properties [13]. The plant has rich source of primary and secondary metabolites including carbohydrates, amino acids, proteins, flavonoids, terpenoids, alkaloids, coumarins, anthraquinones, tannins, saponins, and steroids [6, 13, 14]. The aqueous decoction of the plant was reported to possess antimalarial properties and further confirmed in-vitro against 3D7 strain of *P. falciparum* [6]. The antimicrobial activities of the plant against several bacteria and fungi are well documented [6]. The antidiabetic and antioxidant activities of the plant through bioassay-guided fractionation of the bark were also confirmed [6, 13]. The liver and kidney protective ability of the plant have been confirmed in-vitro with an observed significant reduction in the activities of kidney failure functioning biomarkers [13-15]. Other laboratory confirmed biological activities include anthelmintic [6], anti-inflammatory and analgesic [16], antioxidant [17], anti-trypanocidal [18, 19]. The research study employed the principle of bioassay-guided fractionation on the crude extract of *Afzelia africana* and the determination of its Thin-layer chromatographic (TLC), phytochemical and gas chromatographic profile. The antioxidant, antimicrobial and synergistic properties of the fractions were also evaluated against several bacterial and fungal strains.

Materials and methods

Materials

Reagents: All solvents were purchased from Sigma Aldrich Co. Ltd, Irvine, UK, except the standard drugs. Other chemicals procured include DPPH (Sigma Aldrich, analytical grade, Korea), ABTS (Sigma Aldrich, analytical grade, Korea), DMSO (Sigma Aldrich, analytical grade), artesunate powder (Sigma Aldrich, analytical grade), silica gel 60 (230-400 mesh, Sigma Aldrich, analytical grade, Korea).

Plant material: The bark of *Afzelia africana* was collected from Nsuta (7.0129° N, 1.3783° W) in the Sekyere Central District of the Ashanti region, Ghana. The plant was identified by Mr. Clifford Osafo Asare at the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana. Voucher specimen [KNUST/HM1/2019/SB/008] was deposited at the Herbarium unit for future reference.

Test organisms: The following test organisms were obtained from the Microbiology unit of the School of Basic and Biomedical Sciences, University of Health and Allied Sciences, Ho. The organisms include: methicillin-resistant *Staphylococcus aureus (MRSA)* (NCTC12493), *Staphylococcus aureus (SA)* (NCTC12973), *Escherichia coli (EC)* (NCTC12241), *Streptococcus mutans (SM)* (ATCC700610), *Pseudomonas aeruginosa (PA)* (ATCC4853), *Salmo-*

nella typhi (ST) (ATCC14028), *Klebsiella pneumonia (KP)* (NCTC13440) and *Candida albicans* 1 (CA1) (ATCC90028), fluconazole-resistant *Candida albicans* 2 (CA2) (Clinical) and *Bacillus subtilis (BS)* (ATCC10004).

Extraction of plant material

The collected barks of *A. africana* were cut into pieces, air-dried under shade for three weeks and then pulverized. A 500 g of the pulverized sample was cold-macerated in 90% methanol for three days. The macerated sample was concentrated by using the rotary evaporator (BUCHI Rota vapor R-114). The concentrated sample was further dried, weighed and the yield was 12% w/w (60 g).

Column chromatography and bioassay guided fractionation

Approximately sixty grams (60 g) of the crude methanol extract was adsorbed on 100 g of silica gel with (230-400) mesh size with 100 mL of methanol followed by air-drying. The adsorbed extract was transferred unto the top layer of a 500 g silica gel pre-filled column (open, 45 × 4.5 cm). The solvent systems comprising of petroleum ether, petroleum ether-ethyl acetate, and ethyl acetate were eluted through the column. The procedure yielded 40 fractions that were pooled into four fractions based on a thin layer chromatography (TLC) profile to give fractions F1 (20 mg), F2 (342 mg), F3 (12 mg) and F4 (500 mg) [6].

Phytochemical screening of fractions

The fractions were screened for the presence of flavonoids, phenolics, anthraquinones, terpenoids, steroids, glycosides, coumarins and alkaloids in accordance with previously described methods [6, 20].

GC analysis of fragments

The GC fingerprints of the different fractions were performed using the Perkin Elmer GC Clarus 580 Gas chromatograph interfaced with Perkin Elmer (Clarus SQ 8 S, Australia) Mass Spectrometer. The fractions were partitioned on the DB-5 (ZB-5HTMS; 5% diphenyl/95% dimethylpolysiloxane) fused capillary column with dimensions 30 mm × 0.25 mm ID × 0.25 μm DF. The temperature range was regulated from 70°C to 130°C at 2°C/min and held for 10 min; followed by 130°C to 220°C at 4°C/min and held for 8 min. The temperature was finally adjusted from 220°C to 270°C at 4°C/min and held for 20 min. The diluted fractions were injected in splitless mode at a split-flow rate of 10 mL/min and splitless time of 1 min. The carrier gas consisted of Helium at a flow rate of 1 mL/min with a sample injection volume of 1 uL.

Antioxidant activity

DPPH free radical scavenging activities of fraction: The 2,2-diphenyl picrylhydrazyl (DPPH) scavenging activities of the fractions were examined according to the method previously described by [21] with slight modification. The reaction was performed in 3 mL of methanol containing freshly prepared DPPH (Sigma Aldrich, Korea) and 1 mL of each fractions with concentrations of 1.0, 0.50, 0.25, 0.1, 0.05 and 0.01 mg/mL. The reaction mixtures were protected from light and incubated for 90 minutes at room temperature. The absorbance of the remaining DPPH was recorded calorimetrically at 517 nm with the ultra violet (UV)-visible spectrophotometer (Jenway, Bibby Scientific Ltd, Stone, Staff, UK). The scavenging activities of the fractions were measured as the decrease in absorbance of the DPPH expressed as a percentage of the absorbance of a control DPPH solution without fractions [18, 21]. The procedure was repeated for ascorbic acid in triplicates. The % DPPH scavenging activities of the fractions were determined from the formula with (Ao) and (A) being the absorbances for remaining DPPH for the control and test fractions respectively.

$$\%\ \text{DPPH scavenging activity} = \left[\frac{A_o - A}{A_o}\right] \times 100\%$$

ABTS scavenging activity of fractions: The antioxidant activities (free radical scavenging activity) of the fractions were determined against 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS, Sigma Aldrich, Korea)) by mixing 10 mL of ABTS and 2.4 mM potassium persulphate in order to generate the ABTS radical. The mixture was further diluted in 50 mL of methanol. A 0.80 mL of the solution was then added to 0.20 mL of the prepared fraction concentrations (1, 0.5, 0.25, 0.1, 0.05 and 0.01

mg/mL). The solutions were vortexed and incubated at 30°C for 10 minutes. The absorbance values for the mixtures were read with the UV-visible spectrophotometer (Jenway, Bibby Scientific Ltd, Stone, Staff, UK) at 734 nm for control ABTS (Ao) and the test fractions (A). The free radical scavenging activities for the fractions against ABTS were evaluated by the relation [18].

$$\% \text{ ABTS scavenging activity} = \left[\frac{A_o - A}{A_o}\right] \times 100\%$$

Antimicrobial activtiy

Determination of minimum inhibition concentration (MIC) for fractions: The microbroth dilution technique was carried out as previously described [18] with minor modifications. Exactly 0.10 mL of Mueller Hinton broth were dispensed into all the wells of each of the microtiter plates (Citotest Labware Manufacturing Co. Ltd, Jiangsu, China). A 0.05 mL of each fractions were used to prepare well concentrations ranging from 100-0.01 mg/mL with some selected wells marked as positive (Broth + organism only) and negative controls (Broth with no organism only). The procedure was repeated for the standard antibiotics, ciprofloxacin, tetracycline, fluconazole, nystatin and ketoconazole with prepared concentrations ranging 256-0.125 µg/mL. A 0.10 mL of each of the 0.5 McFarland standardized were added to the test organisms on each column of the wells. The plates were incubated at 37°C for 1-2 days for test organism respectively. The tetrazolium dye was added and after 30 minutes, the MICs were determined by visual analysis.

Determination of minimum bactericidal (MBC) and fungicidal concentration (MFC): In order to verify if the fractions were able to kill the microbial cells (bacteri-/fungi-cidal), the plates were also evaluated for the MBC and MFC. Aliquots from each well from the susceptibility testing assays were transferred to plates containing nutrient agar and then incubated at 37°C for up to two days. The MBC and MFC were then deduced by observation of the presence or absence of growth in the nutrient agar or sabouraud dextrose agar (SDA) [18, 22].

Determination of synergistic effect of fractions with selected antimicrobial agents: The checker board assay method as described by [23] was re-modified to determine the synergistic effects of the fractions with antibiotics, tetracycline and ciprofloxacin against the bacterial strains. The fungal strains were also determined with the standard antifungals, fluconazole, ketoconazole and nystatin against *C. albicans* strains. The tested concentrations for each antibiotic and each test plant samples ranged from 1/32 × MIC to 2 × MIC. The interactions between the fractions and the microbial organisms were evaluated by determining the fraction inhibitory concentration index (FICI) from the formula:

$$\text{FIC index} = \frac{[\text{MIC of antibiotic in combination}]}{[\text{MIC of antibiotic alone}]} + \frac{[\text{MIC of fraction in combination}]}{[\text{MIC of fraction alone}]}$$

The interaction was considered synergistic if the FICI was ≤0.5, partial synergistic if FICI was >0.5 and <1, additive if FICI was = 1, no difference if the FICI was >1 and ≤4, and antagonistic if the FICI was >4.0.

Statistical analysis

GraphPad Prism Software 5.0 was used to analyze all statistical tests. The percentage scavenging activities of the fractions were expressed as mean (n = 3) ± SD (Standard deviation). The data were statistically validated by using one-way Analysis of variance (ANOVA) where the test was considered statistically significant when $P<0.05$. Graphs were plotted using Microsoft (MS) Office Excel 2016.

Results and discussion

Results

Column chromatographic fractionation: The principle of bioassay-guided fractionation technique was employed to generate fractions from cold macerated methanol extract of the bark of *A. africana*. The % yield of the extract (**Table 1**) was evaluated as 12% by evaluating relation, where the mass of crude extract and raw powdered bark were respectively 60 and 500 g.

$$\% \text{ Yield} = \left[\frac{\text{Mass of crude extract}}{\text{Mass of raw powdered bark}}\right] \times 100\%$$

The fractions from the bark of *Afzelia africana* were obtained by mounting the crude extract

Table 1. % Yield of extract

Mass of raw powdered bark (g)	Mass of crude extract (g)	% yield
500	60	12

on a 500 g silica gel (70-230 mesh size). The crude extract after defatting with petroleum ether to generate Fo were eluted with mixtures of solvent systems comprising of petroleum ether and ethyl acetate. The fractionations yielded forty (40) fractions that were pooled into four (4) major fractions based on TLC profile and labelled as F1 (pale yellow), F2 (brown), F3 (brown) and F4 (brown). The solvent system (petroleum ether:ethyl acetate) used in generating the fractions are indicated in parenthesis as F1 (85:15), F2 (75:25), F3 (70:30) and F4 (70:30). The fraction F1 reported three (3) spots with retention factors (Rf) ranging from 0.18-0.55 whilst F2 reported six (6) major spots with Rf values ranging from 0.76-0.98. Moreover, fractions F3 and F4 respectively yielded seven (7) and eight (8) spots with their corresponding Rf values ranging from 0.58-0.98 and 0.25-0.95. The results of the findings are displayed in **Table 2**.

Phytochemical screening: The phytochemical screening experiment (**Table 3**) revealed the presence of flavonoids, phenolics, glycosides, terpenoids and steroids in all the four fractions whilst coumarins were absent. The fraction F1 indicated the absence of alkaloids and anthraquinones whilst F3 and F4 respectively reported the absence of alkaloids and anthraquinones. The observation indicates rich source of phytochemicals in the fractions.

Gas chromatographic (GC) fingerprint: The GC profiles indicating the retention times, peak areas and % composition of the major components in each of the four fractions are presented in **Figures 1-4**. The % compositions of the various components in each of the fractions were evaluated by the relation:

$$\%\ Composition = \left[\frac{\text{Peak area of component}}{\text{Sum of peak areas of all components}}\right] \times 100\%$$

The gas chromatograms revealed the presence of a total of 90, 88, 95 and 90 different secondary metabolites in fractions F1, F2, F3, and F4 respectively. The fraction F1 revealed major peaks with retention times (min) and % compositions at (1.04, 2.3%), (1.52, 24.1%), (75.11, 3.72%), (75.36, 7.09%), (80.02, 4.87%), (80.73, 7.89%) and (119.38, 3.69%) accounting for more than 50% of the total composition. The fractions F2 and F3 respectively reported major peaks accounting for 99.9% with retention times and compositions at (1.37 min., 5.92%), (1.80 min., 39.6%), (1.92 min., 3.14.36%), (1.97 min., 7.77%), (2.03 min., 4.45%) and (2.10 min., 27.83%). Similarly, 6 and 7 major peaks accounting for over 99% of compositions were reported for fractions F3 ((1.36 min., 5.92%), (1.74 min., 33.62%), (1.94 min., 3.24.43%), (1.99 min., 5.15%), (2.02 min., 5.15%), (2.08 min., 25.58%)) and F4 ((1.37 min., 1.34%), (1.79 min., 86.19%), (1.89 min., 3.2.75%), (1.94 min., 1.63%), (2.08 min., 7.99%)) respectively. A summary of the major peaks, their retention time and percentages in each of the fractions are displayed in **Table 4**.

ABTS and DPPH antioxidant activity of fractions: The antioxidant activities of the fractions were determined over concentrations ranging from 1.0, 0.5, 0.25, 0.1, 0.05, and 0.01 mg/mL using ascorbic acid as the reference compound. The report reveals that, at the various concentrations, the fractions exhibited significant scavenging activities against both the ABTS and DPPH radicals. The % range of scavenging activities against ABTS (54-93%) was slightly higher than DPPH (35-76%). Data from the ABTS assay indicates % scavenging activities ranging from 60-100% among all the fractions. The overall grading of their activities was in the order of F1(93-54)>F4(81-58)>F2(74-58)>F3(72-55). At the concentration of 1.0 mg/mL, the fractions F1 and F2 exhibited scavenging activities almost comparable with the ascorbic acid.

The results for the DPPH assay reveal that, at concentration range of 1.0-0.5 mg/mL, fraction F3 reported the highest level of scavenging activity than F1 and then followed by F4 and F2. At the 0.25-0.01 mg/mL concentration range, F3 exhibited the highest level of scavenging activity followed by F1, F4, and F2. The overall grading of scavenging of activity of F3(77-42)>F1(64-46)>F4(55-44)>F2(47-35) is reported in the DPPH assay with overall % scavenging activity ranging from 35-76% for all the concentration ranges. The report in both the ABTS and DPPH assay indicates that, the frac-

Table 2. Thin Layer Chromatographic profile of fractions

Pooled fractions	Colour	Solvent system	No of spots	Retention factor (Rf)
Fo (1-19)		Pet ether	-	-
F1 (20-21)	Pale yellow	Pet ether/Ethyl acetate (85:15)	3	0.18, 0.36, 0.55
F2 (22-30)	Brown	Pet ether/Ethyl acetate (75:25)	6	0.76, 0.78, 0.84, 0.89, 0.93, 0.98
F3 (31-37)	Brown	Pet ether/Ethyl acetate (70:30)	7	0.58, 0.67, 0.78, 0.84, 0.91, 0.96, 0.98
F4 (38-40)	Brown	Pet ether/Ethyl acetate (70:30)	8	0.25, 0.55, 0.64, 0.71, 0.82, 0.85, 0.91, 0.95

Table 3. Phytochemical screening for fractions

Phytochemical test	Fraction 1	Fraction 2	Fraction 3	Fraction 4
Alkaloids	-	+	-	+
Anthraquinone	-	+	+	-
Flavonoids	+	+	+	+
Phenolics	+	+	+	+
Coumarins	-	-	-	-
Glycosides	+	+	+	+
Terpenoids	+	+	+	+
Steroids	+	+	+	+

+: positive, -: Negative.

tions F1, F2, F3, and F4 exhibited % range of scavenging activities of (95-45), (74-35), (72-42) and (80-44) respectively.

Antibacterial and antifungal activity: The antimicrobial activities (bacteriostatic and bactericidal) of fractions F1, F2, F3 and F4 from the bark of *A. africana* were determined by measuring the MIC, MBC and MBC/MIC ratios (**Table 5**) against the microbial strains; *S. mutans* (SM), *S. aureus* (SA), *E. coli* (EC), fluconazole resistant *C. albicans* (CA2), methicillin-resistant *S. aureus* (MRSA), *B. subtilis* (BS), *K. pneumonia* (KP), *P. aeruginosa* (PA), *S. typhi* (ST) and *C. albicans* 1 (CA1) (standard strain). From the context of MIC considerations, fractions F1 (MIC: 2.5-5.0 mg/mL) and F4 (MIC: 0.07-10 mg/mL) exhibited superior inhibitory effects (MICs≤8 mg/mL) at lower MIC values than fractions F2 (MIC≥100.0 mg/mL) and F3 (MIC: 12.5-100.0 mg/mL). The fraction F2 displayed the weakest inhibitory effects against all microbial strains whereas F3 displayed some level of inhibitory effects (MIC≤8 mg/mL) against *E. coli* (MIC: 6.25 mg/mL) and fluconazole resistant *C. albicans* 1 (MIC: 6.25 mg/mL). Furthermore, it could be inferred that, fractions F1 (MBC: 2.5-5.0 mg/mL) and F4 (MBC: 1.25-10.0 mg/mL) exhibited broad spectrum of bactericidal effects at much lower MBC concentrations when compared to fractions F2 (MBC≥100.0 mg/mL) and F3 (MBC: 12.5-100.0 mg/mL) with much higher concentrations. This suggests that higher concentrations of F2 and F3 are required to kill the microbial strains completely. The activities are further corroborated by the MBC/MIC ratios where MBC/MIC≤4 were classified as bactericidal agents. For instance, F1 reported broad spectrum of bactericidal activity (MBC/MIC: (0.6-2.5)) against all the microbial strains at much lower MBC and MIC values. Coincidentally, similar pattern of a broad spectrum of bactericidal activity is reported for fractions F2 (MBC/MIC: (1-2)) against all the microbial strains except *B. subtilis* (MBC/MIC: >64). The fractions F2 (MBC/MIC: ≥1) and F3 (MBC/MIC: (1-4)) reported a broad spectrum of bactericidal effects against all the microbial strains but at much higher MIC and MBC values raising issues of safety concerns. In view of these observations, fractions F1 and F4 were considered to possess higher antimicrobial activity than F2 and F3.

Antibacterial synergistic effects of active fractions: The antibacterial synergistic effects were evaluated by adopting the checkerboard assay technique. The interpretation of the synergistic activity was based on the criteria adopted by Bae et al. using the fractional inhibitory concentration index (FICI) range of 0.5 to 4 where the test was classified as synergistic (S) if FICI≤0.5; partial synergy (PS) if FICI>0.5 to <1.0; additive (AD) if IFCI = 1.0; indifference (I) if FICI>1 to <4.0 and antagonistic if FICI>4.0. The data (**Table 6**) confirmed varying degrees of synergistic activities against the bacterial isolates. For instance, F1 reported a synergistic activity with ciprofloxacin against *S. typhi* and partial synergism against *E. coli*, *K. pneumonia* and *S. aureus*. Moreover, combining F1 with tetracycline showed synergistic activity against *E.*

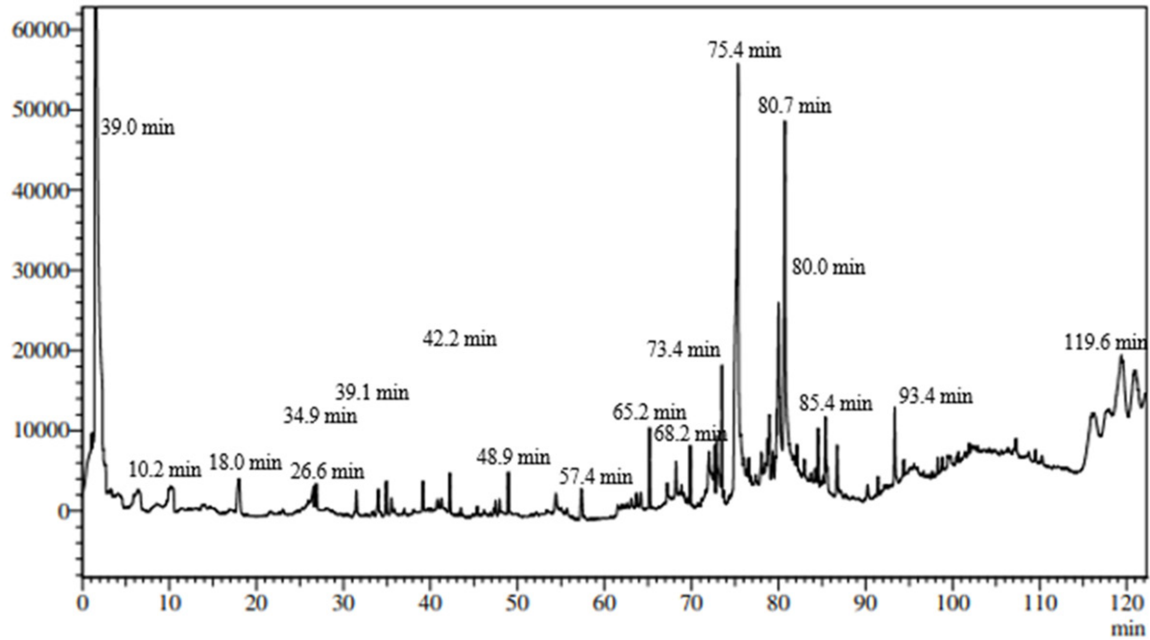

Figure 1. Gas chromatogram of fraction 1.

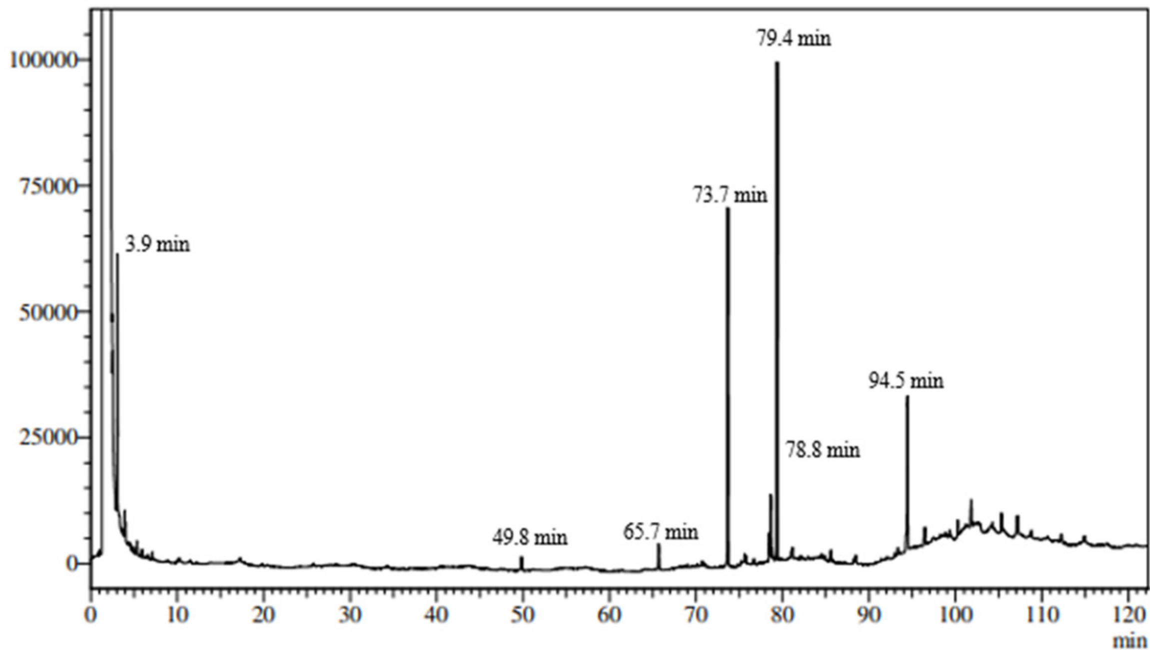

Figure 2. Gas chromatogram of fraction 2.

coli, *K. pneumonia* and *S. typhi*. The fraction F4 also showed synergism with ciprofloxacin against *E. coli*, *K. pneumonia* and *S. typhi*, and partial synergism against *S. aureus*, *S. mutans* and methicillin-resistant *S. aureus*. The fractions further reported synergism with tetracycline against *E. coli*, *K. pneumonia*, *S. typhi* and *P. aeruginosa*. The series of varying synergistic effects reported by fractions F1 and F4 confirms or adds to the broad spectrum of synergistic properties of *A. africana* with the selected standard antibiotics. The analysis of the results further indicates that, fraction F1 exhibited series of indifferent activities with cipro-

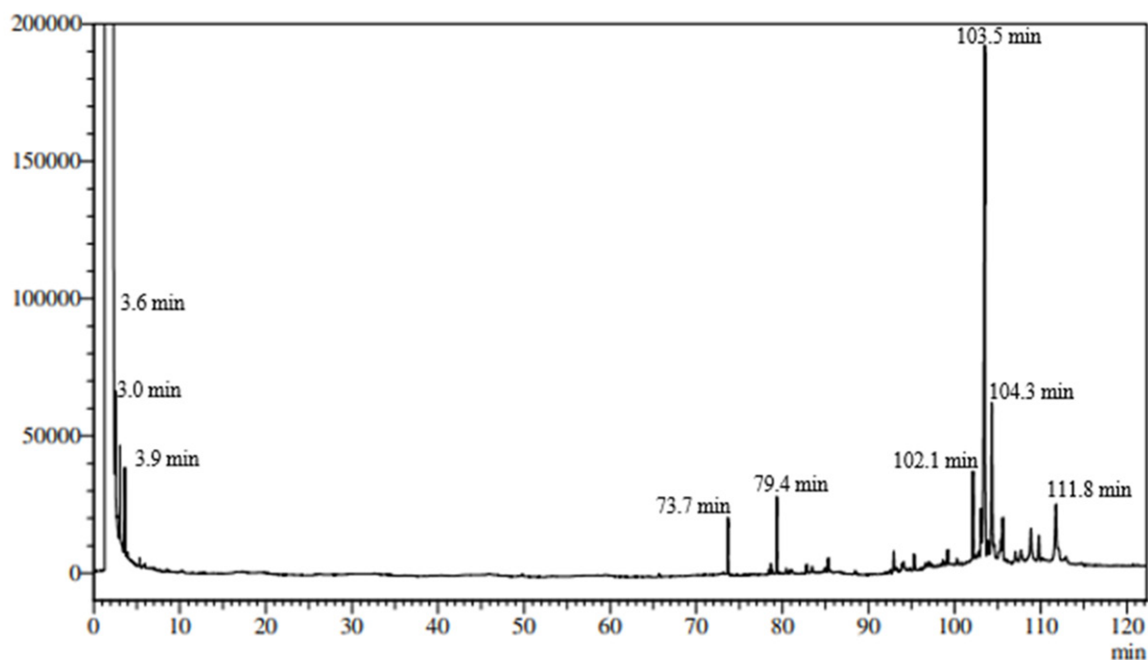

Figure 3. Gas chromatogram of fraction 3.

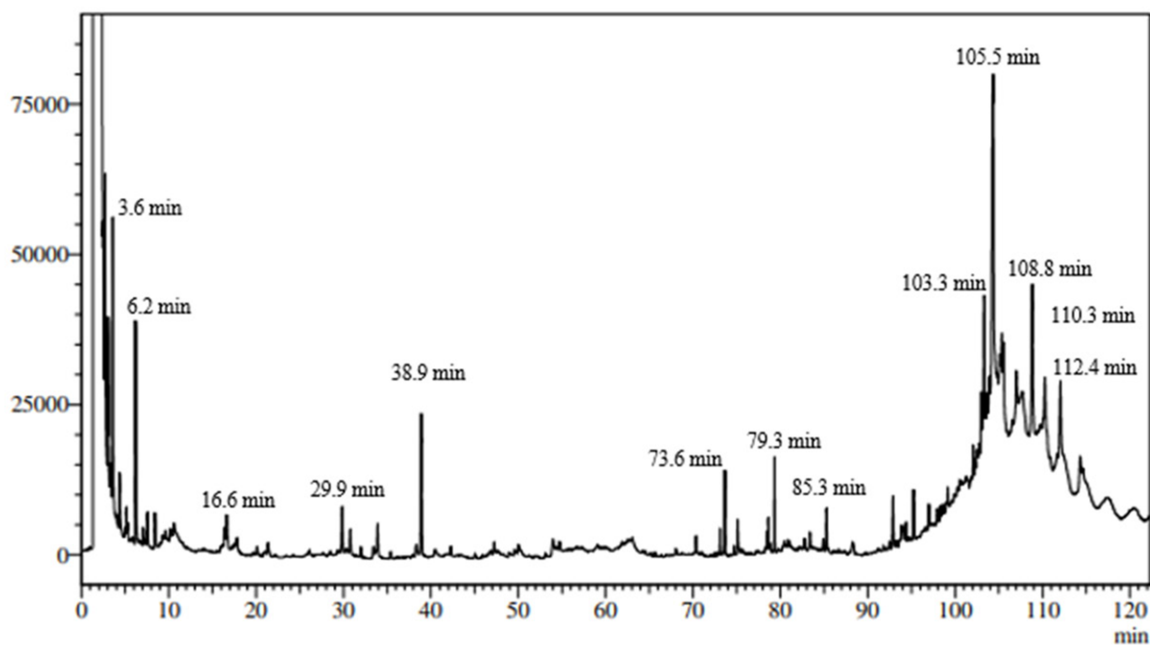

Figure 4. Gas chromatogram of fraction 4.

floxacin (i.e against *S. mutans* and *P. aeruginosa*) and tetracycline (i.e against *S. aureus*, *S. mutans*, methicillin-resistant *S. aureus*, *B. subtilis* and *P. aeruginosa*). Similar activity is observed by F4 with tetracycline against *S. aureus*, methicillin-resistant *S. aureus* and *B. subtilis*. Moreover, whilst the F1 exhibited additive effects with ciprofloxacin against *B. subtilis*, similar activity is observed for F4 with tetracycline against *S. mutans*. Interestingly, none of the fractions exhibited antagonistic effect against the microbial strains.

Table 4. Major peaks and their retention time (Rt) and percentage (%) in fractions

F1		F2		F3		F4	
Rt	%	Rt	%	Rt	%	Rt	%
1.04	2.303	1.37	5.922	1.36	5.915	1.37	1.336
1.52	24.114	1.80	39.593	1.74	33.618	1.79	86.193
72.02	1.243	1.92	14.359	1.94	24.434	1.89	2.747
73.48	1.041	1.97	7.773	1.99	5.145	1.94	1.633
75.11	3.716	2.03	4.453	2.02	5.147	2.08	7.985
75.36	7.089	2.10	27.827	2.08	25.575	103.32	0.003
75.63	1.207	3.07	0.003	3.62	0.003	104.28	0.005
78.79	1.001	65.68	0.001	73.66	0.003	105.12	0.003
80.02	4.867	73.68	0.011	79.35	0.004	105.35	0.003
81.39	7.893	78.49	0.001	103.06	0.005	105.54	0.004
82.42	1.183	78.66	0.002	103.47	0.053	107.01	0.005
85.41	1.068	79.37	0.014	103.89	0.004	107.70	0.004
115.92	1.409	94.27	0.007	104.32	0.018	108.83	0.006
117.62	1.157	94.87	0.001	105.59	0.004	110.28	0.005
119.38	3.691	101.82	0.003	108.85	0.006	112.05	0.004
119.55	1.464	107.16	0.002	111.77	0.010	112.38	0.003
Others	35.553	Others	0.028	Others	0.055	Others	0.061
Total	100.000	Total	100.000	Total	100.000	Total	100.000

Table 5. MIC, MBC and MBC/MIC ratios for fractions

Organism	F1			F2			F3			F4		
	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R	MIC	MBC	R
EC	2.5	>2.5	>1[bc]	100	>100	>1[bc]	6.25	50	4[bc]	5	>5	>1[bc]
CA2	>2.5	2.5	<1[bc]	>100	100	<1[bc]	12.5	12.5	1[bc]	>5	5	<1[bc]
SM	2.5	2.5	1[bc]	>100	100	<1[bc]	25	25	1[bc]	5	5	1[bc]
SA	2.5	2.5	1[bc]	100	>100	>1[bc]	12.5	50	4[bc]	>5	>5	1[bc]
PA	2.5	2.5	1[bc]	>100	>100	1[bc]	100	100	1[bc]	>5	>5	1[bc]
BS	1.25	5	4[bc]	100	>100	>1[bc]	100	100	1[bc]	0.078	>5	>64[bs]
KP	2.5	5	2[bc]	>100	>100	1[bc]	100	100	1[bc]	>5	>5	1[bc]
MRSA	0.625	2.5	4[bc]	100	>100	>1[bc]	25	25	1[bc]	5	10	2[bc]
ST	0.625	2.5	4[bc]	100	>100	>1[bc]	25	100	4[bc]	2.5	5	2[bc]
CA1	>2.5	5	<2[bc]	>100	>100	1[bc]	6.25	50	4[bc]	0.625	1.25	2[bc]

Escherichia coli (EC), fluconazole-resistant *Candida albicans* 2 (CA2), *Streptococcus mutans* (SM), *Staphylococcus aureus* (SA), *Pseudomonas aeruginosa* (PA), *Bacillus subtilis* (BS), *Klebsiella pneumonia* (KP), methicillin-resistant *Staphylococcus aureus* (MRSA), *Salmonella typhi* (ST) and *Candida albicans* 1 (CA1), MIC (Minimum Inhibitory Concentration), MBC (Minimum Bactericidal Concentration), R: MBC/MIC ratio, bc: bactericidal, bs: bacteriostatic, [R≤4 = bactericidal, R>4 = bacteriostatic].

Antifungal synergistic effects of active fractions: The antifungal synergistic activity (**Table 7**) of fractions F1 and F4 revealed varying degrees of synergism against *C. albicans* fungal strains 1 and 2. For instance, the fraction F1 reported synergistic effects with fluconazole against *C. albicans* 2 and indifferent activity against *C. albicans* 1. Additionally, the fraction F1 in combination with ketoconazole showed synergistic activity against *C. albicans* 2 and partial synergism against both fungal strains when combined with nystatin. In reference to fraction F4, varying degrees of synergism were reported against the two fungal strains when combined with fluconazole (indifferent against *C. albicans* 1 and partial against *C. albicans* 2), ketoconazole (indifferent against *C. albicans* 1 and partial synergism against *C. albicans* 2) and nystatin (indifferent synergism against both fungal strains). None of the fractions

Table 6. Synergistic activity of fractions F1 and F4 with antibiotics

Fraction	Test	MIC, FICI and interpretation of synergistic activity against bacterial strains							
F1	Bacteria	EC	KP	SA	SM	ST	MRSA	BS	PA
	MIC (CIPRO)	125.00	125.00	7.81	125.00	3.90	250.00	125.00	125.00
	FIC (CIPRO + F1)	0.771	0.771	0.750	4.000	0.096	0.750	1.000	2.000
	INT	PS	PS	PS	I	S	PS	AD	I
	MIC (TET)	7.81	7.81	7.81	7.81	15.63	7.81	7.81	7.81
	FIC (TET + F1)	0.156	0.156	1.500	1.250	0.375	1.500	1.125	1.125
	INT	S	S	I	I	S	I	I	I
F4	MIC (CIPRO)	125.00	125.00	7.81	125.00	3.90	250.00	125.00	125.00
	FIC (CIPRO + F4)	0.313	0.312	0.750	0.812	0.072	0.750	8.513	2.000
	INT	S	S	PS	PS	S	PS	A	I
	MIC (TET)	7.810	7.81	7.81	7.81	15.63	7.81	7.81	7.81
	FIC (TET + F4)	0.312	0.312	1.513	1.016	0.312	1.513	1.500	0.094
	INT	S	S	I	AD	S	I	I	S

CIPRO: Ciprofloxacin; TET: Tetracycline; INT: Interpretation; FIC: Fractional Inhibitory Concentration Index. INT [S (synergism) if FICI≤0.5; PS (Partial synergy) if FICI>0.5 to <1.0; AD (Additive) if FICI = 1.0; I (indifference) if FICI>1.0 to <4.0 and A (Antagonism) if FICI>4.0].

Table 7. Effects of combined antifungal activity of fractions F1 and F4 with antifungal agents

Agent	MIC		Combination	CA1-1		CA-2	
	CA1	CA2		FICI	INT	FICI	INT
FLC	64	>64	F1 + FLC	2.000	I	0.500	S
			F4 + FLC	8.250	A	0.625	PS
KET	512	512	F1 + KET	0.625	PS	0.258	S
			F4 + KET	2.000	I	0.750	PS
NYX	128	128	F1 + NYS	0.750	PS	0.750	PS
			F4 + NYX	1.250	I	1.5.00	I

FLC: Fluconazole; KET: Ketoconazole; NYX: Nystatin.

exhibited antagonistic effects with the antifungal agents against the fungal strains investigated.

Discussion

The contribution of natural products in health care delivery have been very significant as it has provided wide varieties of secondary metabolites with multiple biological activities [24]. Within the context of drug combination therapy, bioassay-guided fractionation has further provided deeper insight into the biological activities of plants by providing clues into specific fractions with comparative superior activities [6, 10, 18]. The current research was aimed at carrying out bioassay-guided fractionation study on the bark of *A. africana* and its TLC, phytochemical and GC profile. The study consequently generated four fractions which were further investigated for their antioxidant, antibacterial, antifungal and synergistic activities.

The analysis of the column chromatographic fractionation revealed four fractions with retention factors ranging from 0.2-0.98. Literature studies suggest that, retention factors and affinity of phytocompounds for adsorbent medium (stationary phase) correlates with their polarity [25, 26]. It is further established that, a lower Rf value indicates a stronger affinity or interaction between the compound of interest and the stationary phase whilst higher values indicate weaker interactions [26]. Specifically, on a normal phase silica gel TLC plate, lower Rf values could indicate the presence of highly polar groups whilst medium to higher values could indicate intermediate polar to non-polar compounds [26]. The analysis of the fractionation study reveals Rf values ranging from 0.18-0.98 on a normal phase silica gel TLC plate. The results indicate wide structural varieties of secondary metabolites ranging from non-polar, intermediate polar and polar components. The inference is corroborated by the phytochemical profile [6] in **Table 2**, revealing the presence of alkaloids, anthraquinones, flavonoids, phenolics, coumarins, glycosides, terpenoids and steroids.

A key feature of the study involved the determination of the number of compounds present in the four fractions by employing the gas chro-

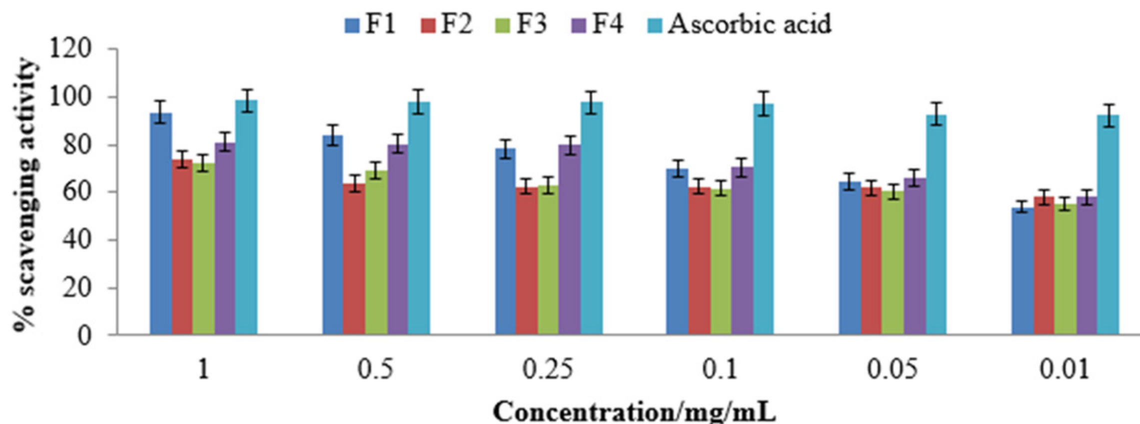

Figure 5. The % ABTS scavenging activities of fractions.

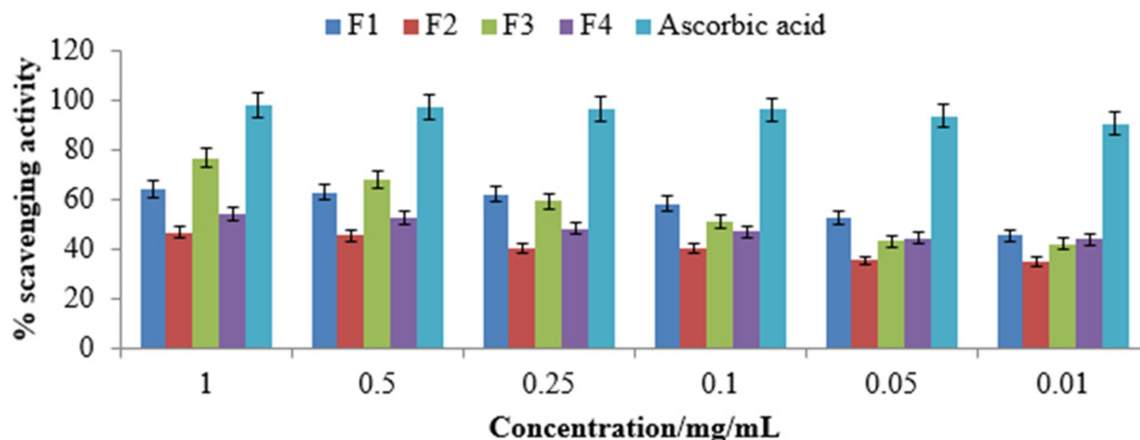

Figure 6. The % DPPH scavenging activities of fractions.

matographic (GC) technique. The technique has shorter analytical time, a higher degree of resolution, accuracy and relatively low detection limits and has been widely utilized in unraveling the presence of bioactive compounds in natural product drug discovery protocols [27-29]. The GC determination of the percentage composition has the clinical significance of helping to standardize plant medicines as part of quality control and assurance protocols [30]. The technique has further helped to check the safety and toxicity profile and the determination of dosage levels of active compounds in herbal formulations [30, 31]. The analysis of the gas chromatogram of the fractions (**Figures 1-4**) revealed the presence of 90, 88, 95 and 90 different secondary metabolites in fractions F1, F2, F3 and F4 respectively. The results of the bioassay-guided fractionation, TLC, phytochemical and the GC profile of the fractions confirms the presence of wide varieties of secondary metabolites in the fractions.

In view of the rich phytochemical constituents identified in the fractions, further studies to ascertain the antioxidant capacity of the fractions were performed by evaluation their scavenging activities against ABTS and DPPH free radicals. The two radicals have been widely applied in evaluating the antioxidant capacities of compounds due to their established mimicking oxygen-nitrogen-centered nature with biological systems [18, 20, 21]. In fact, compounds with good scavenging potentials for these radicals have been applied in the management or treatment of diseases related to oxidative stress [32, 33]. The analysis of the ABTS (**Figure 5**) and DPPH (**Figure 6**) scavenging activities of the fractions reveals that, at concentrations ranging from 1.0-0.01 mg/mL,

the fractions exhibited significant % scavenging capacities against ABTS (54-93%) and DPPH (35-76). The overall ranking of their activities was in the order of F1>F4>F2>F3 and F3>F1>F4>F2 against ABTS and DPPH respectively. Interestingly, the antioxidant capacity of crude extracts from the bark of *A. africana* is extensively reported [16], however, carefully fractionated components or compounds responsible for the reported antioxidant activity is limited. The report of this study has provided further insight into the antioxidant activity of *A. africana* with emphasis on its solvent fractions. In order to account for the reported activities, a careful study of the phytochemical constituents could provide insight. Consequently, all the fractions tested positive for flavonoids and phenolics. As a matter of fact, such phytocompounds have been widely reported to have potent scavenging activities against ABTS and DPPH [32]. The presence of these similar class of secondary metabolites in the fractions could account for the reported activities notwithstanding their variations [34]. The observed differences in the activities of the fractions could be due to the corresponding variations in their phytochemical content as revealed by their TLC, phytochemical and GC profiles. In the quest to elucidate the principle underpinning the mechanism of action of these antioxidant compounds, two mechanistic pathways have been proposed [35]. The first part involves the chain breaking mechanism whereby the antioxidant compound donates electrons to the free radical system [35]. The second mechanism involves the removal of free reactive oxygen and nitrogen species by blocking or decomposing catalysts or agents responsible for initiating free radical generation [35, 36]. Specifically, the mechanism of action underpinning the activities of phenolics and flavonoid compounds are attributed to their ability to bind metal ions [36]. They are also known to scavenge reactive oxygen species and convert hydroperoxides to non-radical species in biological systems [35]. Others have been attributed to their ability to absorb UV radiation or deactivate singlet oxygen [37, 38]. In fact, a mechanistic study by Platzer *et al.* [38] shows that, the antioxidant capacity of such compounds is influenced by the number of phenolic groups in their structure. They further act as antioxidants by donating the protons (H) in their phenolic systems [35, 38]. The generated species subsequently react with reactive oxygen and nitrogen species in a termination reaction, leading to the breakage of the cycle of the generation of new radicals [39, 40]. Further to the mechanism of these phytochemicals, they have been known to inhibit free radical generation by the inhibition of lipid peroxidation, chelation, and reduction of metal ions [40]. Mechanistically, flavonoids and phenolic compounds, are known to regulate the key biomarker, NrF2 involved in the production of free radicals [41, 42]. The regulation could therefore subside the generation and propagation of free radicals. The series of accounts could therefore contribute to the promising antioxidant activities of the fractions.

The antimicrobial properties of the fractions were further determined by evaluating their bacteriostatic and bactericidal effects. The bacteriostatic and bactericidal effects were determined by evaluating the minimum inhibitory (MIC) and bactericidal (MBC) concentrations and their ratios (MBC/MIC) consistent with good microbial assay practices [22, 43, 44]. Furthermore, MBC/MIC≤4 were considered as bactericidal whilst MBC/MIC>4 were considered bacteriostatic [43]. The analysis of the results indicates that, fractions F1 and F4 were considered to possess higher antimicrobial activity than F2 and F3 and hence could serve as antimicrobial agents or synergistic boosters for standard antimicrobial agents. The reported activities by the fractions against *S. aureus, B. subtillis* and *E. coli* corroborates those reported by the crude extract of *A. africana* in literature [6, 13]; giving scientific credence to the utilization of the plant as an antimicrobial agent. In several literatures, antimicrobial activities of plant extracts are attributed to their rich secondary metabolites and synergistic interactions [45]. The activities have been traced to the presence of phytocompounds such as phenolics, flavonoids, alkaloids, steroids, coumarins, tannins, etc [45-47]. The underlying mechanistic activities of the various phytochemical classes are attributed to the presence of poly-phenolics (e.g. phenolic, flavonoids, coumarins, tannins); ether/lactones (coumarins, ellagic acid); benzo-1-pyran-4-quinone (flavonoids); sapogenin (saponins, steroids, terpenes) and isoprene (terpenoids) structural motifs [48-50]. Furthermore, structural motifs responsible for the antimicrobial

activities of alkaloids have been traced to the presence of pyridinyl (e.g. piperine), piperidinyl, quinolinyl, indolyl, pyrrolidinyl, quinazoline, isoquinoline, glyoxaline, lupinane, tropan, phenanthridine and imidazoline [51]. They have been known to exhibit broad spectrum of biological activities such as antibacterial (both gram positive, gram negative bacterial, and drug resistant strains), antiviral, antifungal and antioxidant [49, 50]. The presence of these structural moieties is implicated in the inhibition of bacterial growth by interference or inhibition of bacterial cell wall and protein synthesis as well as increased cell wall permeability [48-51].

In view of the promising antimicrobial activities reported for F1 and F4, further tests to determine their synergistic activities with standard antibacterial agents such as ciprofloxacin and tetracycline were performed. The experiment was necessary as it could provide candidates that could serve as new discoveries or act synergistically to boost drug-resistant candidates. The interpretation of the synergistic activity was based on the criteria adopted by Bae et al. using the fractional inhibitory concentration index (FICI) range of 0.5 to 4 where the interaction was classified as synergistic (S) if FICI≤0.5; partial synergy (PS) if FICI>0.5 to <1.0; additive (AD) if IFCI = 1.0; indifference (I) if FICI>1 to <4.0 and antagonistic if FICI>4.0 [43, 45]. The analysis shows that the two active fractions (F1 and F4) exhibited varying degrees of synergistic activities ranging from complete synergism, partial, additive and indifferent. The wide array of synergistic activties reported by fractions F1 and F4 adds to the broad spectrum of synergistic properties of *A. africana* with the selected antibiotics investigated [13]. The contribution of phytochemicals to the antimicrobial activities of plant medicines is widely reported [52] and hence could account for the reported synergistic activities in this study. The mechanistic study of the contribution of phytochemicals to the synergistic effect of antibiotics is well documented [53, 54]. The activities have been linked to their ability to facilitate or enhance antibiotic interaction with the target site of the pathogen. The resultant effect has helped to curb the emergence of resistance [53, 54]. For instance, flavonoids and phenolic compounds containing series of polyphenolic systems have been known to have synergistic effects with ciprofloxacin against methicillin-resistant *Staphylococcus aureus* [53, 54]. In other studies, several alkaloid derivatives such as piperine, indoles and indirubicin yielded synergistic effects with ciprofloxacin and tetracycline against *S. aureus* through the resistance-nodulation-division (RND) efflux pump pathway [50]. The synergistic effects of flavonoid derivatives (e.g. epigallocatechin gallate and epicatechins) with tetracycline and other antibiotics have been reported against several bacterial strains [50]. It is instructive to note that, the mechanism of action of these phytochemicals proceeded through the major facilitator superfamily (MSF) and the multi-drug resistant (MDR) efflux pump pathway. They are also known to enhance the activities of antibiotics by disintegrating the oily outer membrane of bacteria, thereby increasing their membrane permeability for antibiotic activity [50, 53, 54]. These accounts therefore corroborate the synergistic effects reported by fractions F1 and F4 against *S. aureus*, methicillin-resistant *S. aureus* and the other microbial strains.

The antifungal synergistic effects of the fractions F1 and F4 were evaluated against *Candida albicans* 1 and 2 strains through the checkerboard assay method [23]. The analysis of the results reveals that, the two fractions reported varying degrees of synergistic effects ranging from complete synergism, partial, additive and indifferent when combined with fluconazole, ketoconazole and nystatin. However, only fraction F1 reported complete synergism against fluconazole resistant *Candida albicans* when combined with fluconazole and ketoconazole. The myriad of antibacterial and antifungal activities of the fractions further re-enforces the vast antimicrobial activities reported for *A. africana* [13, 18] against several bacterial and fungal infections. The synergistic effects of phytocompounds with antifungal agents have been recognized as one of the effective tools used in fighting fungal infections and their accompanying drug resistance [55]. Several synergistic effects of phytocompounds with antifungal agents against *C. albicans* are reported. For instance, the flavonoid derivatives such as curcumin and chalcones in combination with antifungal agents such as fluconazole, ketoconazole and nystatin have been reported by Aboody *et al*. against *C. albicans* [56]. The reported synergistic activities of F1

and F4 against *C. albicans* corroborates the report by Aboody *et al*. since they contain similar phytochemical classes (**Table 2**). These phytocompounds exhibit their synergistic effects mechanistically by decreasing the ability of cells to efflux out the antibiotics. The effects therefore enhance the permeability, bioavailability and effectiveness of antibiotics at their target sites [56]. They are also known to induce oxidative stress in the fungal strains by activating reactive oxygen species, superoxide dismutase, catalase and oxidoreductases [32]. The flavonoids are also known to perform their synergistic effects by the alteration of the cell wall and cell-cell adhesion communication in the fungal strain [57]. The cascading effect leads to the disruption of genes expression responsible for biofilm formation in the fungal strain [57], thereby exposing the pathogen to antibiotic attack.

The research has brought to the fore, the rich experimental data on the four fractions from the root bark of *A. africana* and their reported biological activities. The future expectation of the research study is to explore the possible formulation and standardization of herbal formulas for the treatment of microbial infections as well as diseases involving oxidative stress. The other future prospect of the study is to further fractionate the fractions and isolate active compounds responsible for their biological activities.

Conclusion

Bioassay-guided fractionation of the methanol extract of *Afzelia Africana* led to the isolation of four fractions. The fractions exhibited promising antioxidant activities with significant scavenging percentages ranging from 30-100% against ABTS and DPPH radicals. The factions reported varying degrees of antimicrobial activities against *S. mutans*, *S. aureus*, *E. coli*, fluconazole-resistant *C. albicans*, methicillin-resistant *S. aureus*, *B. subtilis*, *K. pneumonia*, *P. aeruginosa, S. typhi* and *C. albicans* (standard strain). The fractions further produced promising synergistic effects against both resistant and non-resistant bacterial and fungal isolates when combined with standard antibacterial (ciprofloxacin and tetracycline) and antifungal (fluconazole, ketoconazole, and nystatin) agents. The report confirms the ethnobotanical utilization of *Afzelia africana* for the treatment of both infectious and non-infectious diseases.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Bright Yaw Vigbedor, Department of Basic Sciences, School of Basic and Biomedical Sciences, University of Health and Allied Sciences, PMB 31 (00233), Ho, Ghana. Tel: 233245052908; E-mail: ybvigbedor@uhas.edu.gh

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