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Solvent–solvent fractionations of *Combretum erythrophyllum* (Burch.) leave extract: Studies of their antibacterial, antifungal, antioxidant and cytotoxicity potentials

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ABSTRACT

Objective: To evaluate the biological activities of *Combretum erythrophyllum* (*C. erythrophyllum*) leaf extracts against infectious diseases' pathogenesis and their cytotoxicity potentials.
Methods: Powdered leaf material (300 g) of *C. erythrophyllum* was extracted (1:10 w/v) using acetone to obtain the crude extract. Liquid–liquid fractionation was performed on the crude acetone extract (30 g) using solvents of different polarity. The bioautographic method was used to detect the inhibition of bacterial and fungal growth by active compounds present in the crude and fractions. The extracts were then tested on bacterial strains: *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*; fungal strains: *Candida albicans* (*C. albicans*), *Cryptococcus neoformans*, and *Aspergillus fumigatus*, by microtitre dilution method for MIC determination.
Results: The extracts MIC values ranged between 0.08 and 2.50 mg/mL against the tested pathogens. Water fraction had the highest activity against bacteria strains, while the fungal assay revealed crude acetone extract and ethyl acetate fraction to be active against *C. albicans* (1.25 mg/mL), dichloromethane extract against *C. albicans* and *A. fumigatus* (0.16 mg/mL). Extract fractions showed a good antioxidant activity via DPPH, ABTS and hydroxyl radical scavenging assays, in the order: ethyl acetate > water > acetone > dichloromethane > hexane. The toxicity level of crude extract and fractions evaluated in Vero monkey kidney cells ranged from 34 to 223 μg/mL, while doxorubicin (IC_{50} = 7.19 μg/mL) served as the positive control.
Conclusions: It can be concluded that the extracts of *C. erythrophyllum* are safe for medicinal use in folk medicine for treating infectious and stress related diseases.

1. Introduction

Medicinal plants are well known natural sources of therapeutic agents used for the treatment of various diseases. About 20 000 plant species have been documented to be valuable for medicinal purposes by World Health Organization (WHO) [1]. Amongst medicinal plants use in South African, species of *Combretum* features prominently as agent for treating infectious diseases such as diarrhea (*Combretum imberbe*, *Combretum vendae*), malaria (*Combretum ghasalense*), stomach disorders (*Combretum molle*) and coughs [*C. molle*, *C. imberbe*, *Combretum erythrophyllum* (*C. erythrophyllum*)] [2].

Infectious diseases are the serious problem and momentous cause of morbidity and mortality worldwide, particularly in the developing countries. This accounts for approximately 50% of all deaths, where access to health care is inadequate and as high as 20% of deaths in the developed countries [3]. Despite the milestones reached in microbiology with the discovery and

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application of antibiotics, and control of microorganisms. The sporadic incidents of epidemics due to drug-resistant microorganisms and emergence of unknown disease-causing microbes still pose an enormous threat to the healthcare system. Some of the chemotherapeutic agents currently in use are toxic with associated adverse side effects [4]. Therefore, the need for new anti-infective and chemotherapeutic agents against various diseases pathoaetiologies, which are highly effective, low toxicity with minor environmental impact. Plant-based medicines have many traditional claims including the treatment of ailments of infectious origin.

In South African traditional medicine, many plant species are used to treat or serve as a prophylaxis against various forms of the disease (infectious and non-infectious) [5]. Within Africa context, medicinal plant species are traded for use in traditional medicines, of which most are from ethno-pharmacological guide [6]. The sustainable use and management of medicinal plants are of the considerable challenge to all stakeholders. The stem, bark, and roots of many medicinal plants are being harvested and traded in an unsustainable manner that may lead to accelerated death of the tree, the source of medication. Evaluation and validation of bioactivity of the leaf extracts as a possible substitute for the use of stem, bark, and roots provide a viable option for the conservation of medicinal plants.

C. erythrophyllum is a member of Combretaceae family, widely used for the treatment of venereal diseases [7]. Roots are used as a purgative while dried and powdered gum can be applied to sores [8]. *C. erythrophyllum* is widely distributed in the Southern Africa region, mostly found in South Africa along the coast in the Eastern Province, through Kwazulu-Natal. At Northern South Africa, this plant species can be found in Mpumalanga, Limpopo Province, Gauteng and the Eastern parts of North West, Zimbabwe, Swaziland, Mozambique and slightly into the eastern parts of Botswana [9].

Seven antibacterial phenolic compounds identified by Martini *et al.* [10] including: four flavonols (5,6,4′-trihydroxyflavononol (kaempferol)), 5,4′-dihydroxy-7-methoxyflavonol (rhamnocitrin), 5,4′-dihydroxy-7,5′-dimethoxyflavonol (rhamnazin) and 7,4′-dihydroxy-5,3′-dimethoxyflavonol (quercetin-5,3′-dimethylether). The three flavones: 5,7,4′-trihydroxyfavone (apigenin), 5,4′-dihydroxy-7-methoxyflavone (genkwanin) and 5-hydroxy-7,4′-dimethoxyflavone were isolated from *C. erythrophyllum*. The compounds exhibited good activity against *Vibrio cholerae* and *Enterococcus faecalis* (*E. faecalis*), with MIC value of <100 μg/mL. Rhamnocitrin and quercetin-5,3′-dimethylether inhibited *Micrococcus luteus* and *Shigella sonei* with MIC value of 25 μg/mL [10].

Oxidative stress occurs when there is a slight imbalance in favor of ROS/RNS and this may occur in numerous circumstances. Such include disease or malnutrition where there are insufficient micronutrients to meet the needs of the antioxidant defenses [11]. However, some plant extracts, other materials and products derived from the plant have been associated with quenching of free radicals, thereby possessing antioxidant potentials. Epidemiological studies have shown that many of the antioxidant compounds also possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial or antiviral activities to a greater or lesser extent [12]. From a report by Martini *et al.*, one antioxidant compound isolated from *C. erythrophyllum* that is 5-hydroxy-7,4′-dimethoxyflavone+, showed the weakest activity [10].

Hence, the study aimed at evaluating the biological activities of *C. erythrophyllum* leaf extracts against infectious diseases' pathogenesis and studied the antioxidant, cytotoxic potentials of the hexane, water, dichloromethane and ethyl acetate fractions.

2. Materials and methods

2.1. Plant collection and treatment

C. erythrophyllum (Burch.) leaves were collected from a tree within the University of Pretoria Botanical garden (Onderstepoort campus), Gauteng Province, Republic of South Africa. The plants were taxonomically identified by Prof. J. N. Eloff, the University of Pretoria and the voucher specimen CL Bredenkamp 1542 was deposited at the National Herbarium in Pretoria. Collected leaves were washed with distilled water to remove debris, dried at room temperature for weeks. The dried leaves were then pulverized into powder using Macasalab mill (model 200 Lab), sieved using 2 mm mesh and then stored in a dry, airtight container for further usage.

2.2. Preparation of leaf material and extraction

Leaf material was extracted (1:10 w/v) using acetone (Merck, South Africa) as extractant with constant shaking on Labotec model 20.2 shaking machine for 6 h. The supernatant was removed from the residue by filtration using Whatman No.1 filter paper. This process was repeated 3 times to exhaustively extract the plant material and the extracts were combined. The solvent was removed under vacuum using a rotary evaporator. The acetone extract was then dissolved in 70% acetone and 30% water in a separating funnel, equilibrated and extracted first with hexane, dichloromethane and ethyl acetate (Merck, South Africa) successively to produce fractions of different polarities. Fractions were concentrated on a rotary evaporator, followed by air drying at room temperature. The residual water fraction was freeze-dried. The fractionation protocol is presented in Figure 1.

2.3. Bioautography

The bioautographic procedure described by Begue and Kline [13] was used to determine the number of active compounds

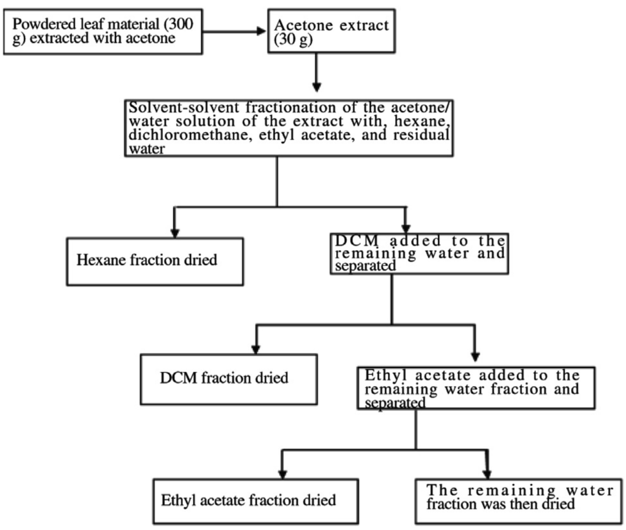

Figure 1. Protocol for the solvent–solvent fractionation of acetone leaf extract components of *C. erythrophyllum*.

in the acetone crude extract, hexane, dichloromethane, ethyl acetate fractions and water residue of *C. erythrophyllum*. Merck TLC F254 plates were loaded with about 10 μL of the extracts. The prepared plates were then developed in Hexane: Ethyl acetate (v/v, 60:40). The chromatograms were dried for 2 d at room temperature under a stream of air to remove solvents and sprayed with a concentrated suspension of actively growing cells of gram-positive: *Staphylococcus aureus* (*S. aureus*) (ATTC 29213), *E. faecalis* (ATTC 29212), gram-negative: *Escherichia coli* (*E. coli*) (ATTC 27853), *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATTC 25922), and fungi: *Candida albicans* (*C. albicans*), *Cryptococcus neoformans* (*C. neoformans*) and *Aspergillus fumigatus* (*A. fumigatus*), followed by incubation at a relative humidity of 37 °C overnight (18 h) in a tight chamber at 100% relative humidity. After 18 h, the plates were further sprayed with 2 mg/mL of *p*-iodonitrotetrazolium (INT) violet (Sigma–Aldrich, South Africa) solution and then incubated at 37 °C for 1 h. Inhibition of bacterial growth was indicated by clear zones on the chromatogram. Microbial growth causes reduction of the colorless tetrazolium salt to a red formazan [13].

2.4. Minimum inhibitory concentration (MIC)

MIC of active extracts was studied by using broth microdilution method with slight modifications. A concentration of 10 mg/mL of the plant extracts, gentamicin (bacteria positive control) and amphotericin B (fungal positive control) were prepared by dissolving in acetone within a vial. It was then placed in the shaker until all the extracts were properly dissolved giving rise to a homogeneous solution. And 100 μL of distilled water was added to the 96-well microplates using a multichannel micropipette. The plant extracts (100 μL) were added to the first well of the column and serially diluted by two-fold together with gentamicin and amphotericin B (positive controls). The freshly cultured bacteria were prepared from an overnight culture and diluted with fresh pre-sterilized MH broth (1:100). The bacterial culture (100 μL) was added to the test sample in each well of the microtitre plate. The organism and the extract mixtures were incubated for 16 h at 37 °C. Organism growth was detected after the addition of 40 μL of 0.2 mg/mL INT (Sigma–Aldrich, South Africa) solution followed by incubation for 30 min at 37 °C. The color change from yellow to purple indicated the presence of microbial growth. All the experiments were performed in triplicate [14].

Bacterial strains were obtained from Department of Veterinary Sciences, the University of Pretoria at Onderstepoort. The following strains were used in this study, gram-positive: *S. aureus* (ATTC 29213), *E. faecalis* (ATTC 29212), gram-negative: *E. coli* (ATTC 27853), *P. aeruginosa* (ATTC 25922), and fungal strains: *C. albicans, C. neoformans* and *A. fumigatus*.

2.5. Total activity of the extracts

Total activity gives an indication of the volume to which the bio-active compounds extracted from 1 g of plant material can be diluted and still can inhibit the growth of a microorganism.

In this study, the total activity of *C. erythrophyllum* extracts with respect to their amount extracted was calculated as follows:

$$\text{Total activity}\left(\frac{\text{mL}}{\text{mg}}\right) = \text{Amount extracted from 1 mg}\Big/\text{MIC}\left(\frac{\text{mg}}{\text{mL}}\right)$$

2.6. Evaluation of antioxidant activity

The free radicals scavenging potential were evaluated using the following assays: ABTS assay, DPPH assay, hydroxyl radical scavenging.

2.6.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity

DPPH is used as a free radical to determine the antioxidant potential of natural compounds. The degree of its discoloration is attributed to the hydrogen donating ability of the test compound. DPPH scavenging activity of *C. erythrophyllum* acetone extract, hexane, dichloromethane, ethyl acetate fractions and water residue were carried out per method described previously [15]. Two milliliters of each extract (0–5 mg/100 mL CH_3OH) were added to 2 mL of DPPH (2 mg/100 mL CH_3OH) respectively. The mixtures were incubated at room temperature in the dark for 30 min. The absorbance of the reaction mixtures was measured at 516 nm using spectrophotometer (UV–Vis model: T80+, PG Instruments Ltd). Ascorbic acid (Sigma–Aldrich, Johannesburg, South Africa) at concentration of 0.5 mg/100 mL, was used as positive control. The percent inhibition obtained for the sample extracts and standard were expressed as half maximal inhibitory concentration (IC_{50}) values, mg/mL (the concentration of the sample required to reduce the initial DPPH concentration by 50%).

2.6.2. ABTS assay

The 96 well microtitre plates were filled with 40 μL of methanol. The *C. erythrophyllum* extracts (40 μL) were added to all the wells followed by 160 μL of $ABTS^+$ solution (working solution was prepared by co-dissolving 38.4 mg of ABTS and 6.6 mg potassium persulfate ($K_2S_2O_8$) in 10 mL of water and allowed to stand in the dark environment to form stable radical cation for 16 h) and the absorbance was measured immediately using microplate reader (EMax® Plus, Molecular Devices) at a wavelength of 734 nm. The plate reading was repeated after 7 min [16]. Quercetin (Sigma–Aldrich, Johannesburg, South Africa) was used as the standard. The percent inhibition obtained for the sample extracts were also expressed as IC_{50} values (mg/mL).

2.6.3. Hydroxyl radical scavenging assay

The 96 well microtitre plates were filled with 66 μL of methanol. The plant extracts (66 μL) were then added to the first well and serial dilution was then performed. $FeSO_4$, H_2O_2 and salicylic acid (120 μL) were added to each well. The 96 well microtitre plates were incubated in an oven at 37 °C for 30 min. The absorbance was measured using microplate reader (EMax® Plus, Molecular Devices) at a wavelength of 532 nm [17]. Ascorbic acid (Sigma–Aldrich, Johannesburg, South Africa) was used as reference standard. Hydroxyl radical scavenging activity of the extracts was expressed in IC_{50} values, mg/mL.

2.7. Evaluation of cytotoxicity using (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (MTT) assay

Viable cell growth after incubation with the test compounds was determined using the tetrazolium-based colorimetric assay (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide)

(MTT assay) as previously described by Mosmann [18]. Vero monkey kidney cell lines of a subconfluent culture obtained from the cell culture collection of the Department of Tropical Diseases (University of Pretoria), were harvested and centrifuged at 200 × *g* for 5 min and re-suspended in growth medium to obtain (5 × 10^4) cells/mL. The growth medium used was minimal essential medium (MEM, Highveld Biological, South Africa) supplemented with 0.1% gentamicin (Virbac, Centurion, South Africa) and 5% fetal calf serum (Highveld Biological, Sandton, Modderfontein, South Africa). A total of 200 μL of the cell suspension was pipetted into each well of columns 2–11 of a sterile 96-well microtitre plate. Growth medium (200 μL) was added to wells of columns 1 and 12 to minimize the edge effect and maintain humidity. The plates were incubated for 24 h at 37 °C in a 5% CO_2 incubator until the cells were in the exponential phase of growth. The MEM was aspirated from the cells and replaced with 200 μL of test fractions at different concentrations. The cells were disturbed as little as possible during the aspiration of the medium and addition of the test compound. Each dilution was tested in triplicate. The microtitre plates were incubated at 37 °C in a 5% CO_2 incubator for 2 d with the test compound and extracts. Untreated cells as negative control and positive control (Doxorubicin Hydrochloride, Adriblastina CSV, Pfizer, Johannesburg, South Africa) were included. After incubation, 30 μL MTT (Sigma, a stock solution of 5 mg/mL in PBS, Sigma–Aldrich, Johannesburg, South Africa) was added to each well and the plates were further incubated for 4 h at 37 °C. After incubation with MTT, the medium in each well was carefully removed without disturbing the MTT crystals in the wells. The MTT formazan crystals were dissolved by adding 50 μL DMSO to each well. The plates were shaken gently until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (Versamax, Molecular Devices) at a wavelength of 570 nm. The wells in column 1, containing medium and MTT but no cells were used to blank the plate reader. The LC_{50} values were calculated as the concentration of the test compound resulting in a 50% reduction of absorbance compared to untreated cells [18].

2.8. Statistical analysis

All experiments were completed in triplicate to ensure reproducibility. The concentration of each fraction was obtained from the calibration functions of each replicate and results obtained were reported as mean values ± standard deviation (mean ± SD), $n = 3$.

3. Results

3.1. Material extraction

Figure 1 illustrated the solvent–solvent extraction from the powdered leaf material: crude acetone extract (30 g) of *C. erythrophyllum* leaves using different solvents. Dichloromethane fraction extracted the highest mass (5.66 g) and the lowest mass was found in hexane fraction (0.66 g). The crude acetone amounts extracted from the leaves of *C. erythrophyllum* was 30 000 mg and the solvent fractions extracted included dichloromethane (5 660 mg), hexane (660 mg) ethyl acetate (3 460 mg) and water (3 480 mg). It could be observed that dichloromethane gave the highest quantity of extractable material from the crude acetone extract while hexane extracted the lowest quantity.

3.2. Bio-assays

The bioautography assay was used to evaluate the antibacterial and antifungal activity of compounds present in the *C. erythrophyllum* extracts as displayed in Figure 2. The antimicrobial effects of the acetone (ACET), hexane (HEX), dichloromethane (DCM), water (H_2O) and ethyl acetate (EA) fractions against the *S. aureus* (Sa), *E. faecalis* (Sf), *P. aeruginosa* (Pa), and *E. coli* (Ec) (bacterial) and *C. neoformans* (Cn), *C. albicans* (Ca), *A. fumigatus* (Af) (fungal) were evaluated.

The MIC values for the *C. erythrophyllum* extracts in this present study ranged between 0.08 and 2.50 mg/mL for all the tested pathogens: *S. aureus*, *E. faecalis*, *P. aeruginosa*, and *E. coli* (bacterial) and *C. neoformans*, *C. albicans*, *A. fumigatus* (fungal). The values are presented in Tables 1 and 2. The extracts showed a significant activity against microorganisms, both gram-positive and gram-negative bacteria with MIC values in the range of (0.080 ± 0.005)–(2.500 ± 0.260) mg/mL. Dichloromethane and hexane fractions gave (0.320 ± 0.220)–(1.250 ± 0.180) mg/mL, acetone and ethyl acetate extracts showed a MIC value of (0.080 ± 0.005)–(1.250 ± 0.040) mg/mL against bacterial strains. Water extracts inhibited fungal strains within the range (0.080 ± 0.008)–(0.620 ± 0.015) mg/mL.

The total activity of all fractions and crude acetone with the different bacterial and fungal strains were listed in Table 3. Acetone extract had the highest average total activities (114 mL/mg) with respect to all the tested strains, followed by dichloromethane extract (27.20 mL/mg) while hexane gave the least average activities (2.62 mL/mg).

In vitro antioxidants activities of crude acetone extract, dichloromethane, hexane, ethyl acetate, water fractions of *C. erythrophyllum* leaves were evaluated by different methods: 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay and hydroxyl radical scavenging assay as presented in Table 4. The fractions scavenged DPPH radical in the range (0.043 ± 0.010)–(0.131 ± 0.003) mg/mL; ABTS radical (0.018 ± 0.006)–(0.256 ± 0.006) mg/mL; hydroxyl radical ranged from (0.015 ± 0.003)–(0.048 ± 0.040) mg/mL. Ethyl acetate and water (Table 4) exhibited high scavenging actions (IC_{50}, mg/mL) against the studied radicals. Ethyl acetate: hydroxyl radical (0.017 ± 0.007) > $ABTS^+$ radical (0.040 ± 0.010) > DPPH radical (0.043 ± 0.010), while water inhibited $ABTS^+$ radical (0.018 ± 0.006) > hydroxyl radical (0.026 ± 0.005) > DPPH radical (0.058 ± 0.020).

The tested extracts exhibited high cytotoxic activity against Vero cell lines within the range 34.80–223.10 μg/mL with values higher than the standard doxorubicin [LC_{50} = (7.190 ± 0.966) μg/mL] used as positive control. LC_{50} was lethal concentration 50, MTT = 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide. In addition, the cytotoxicity average values and standard deviation of crude extract, dichloromethane fraction, ethyl acetate fraction, water fraction were (34.80 ± 0.025), (36.60 ± 0.011), (94.70 ± 0.001), and (223.1 ± 0.033) μg/mL respectively.

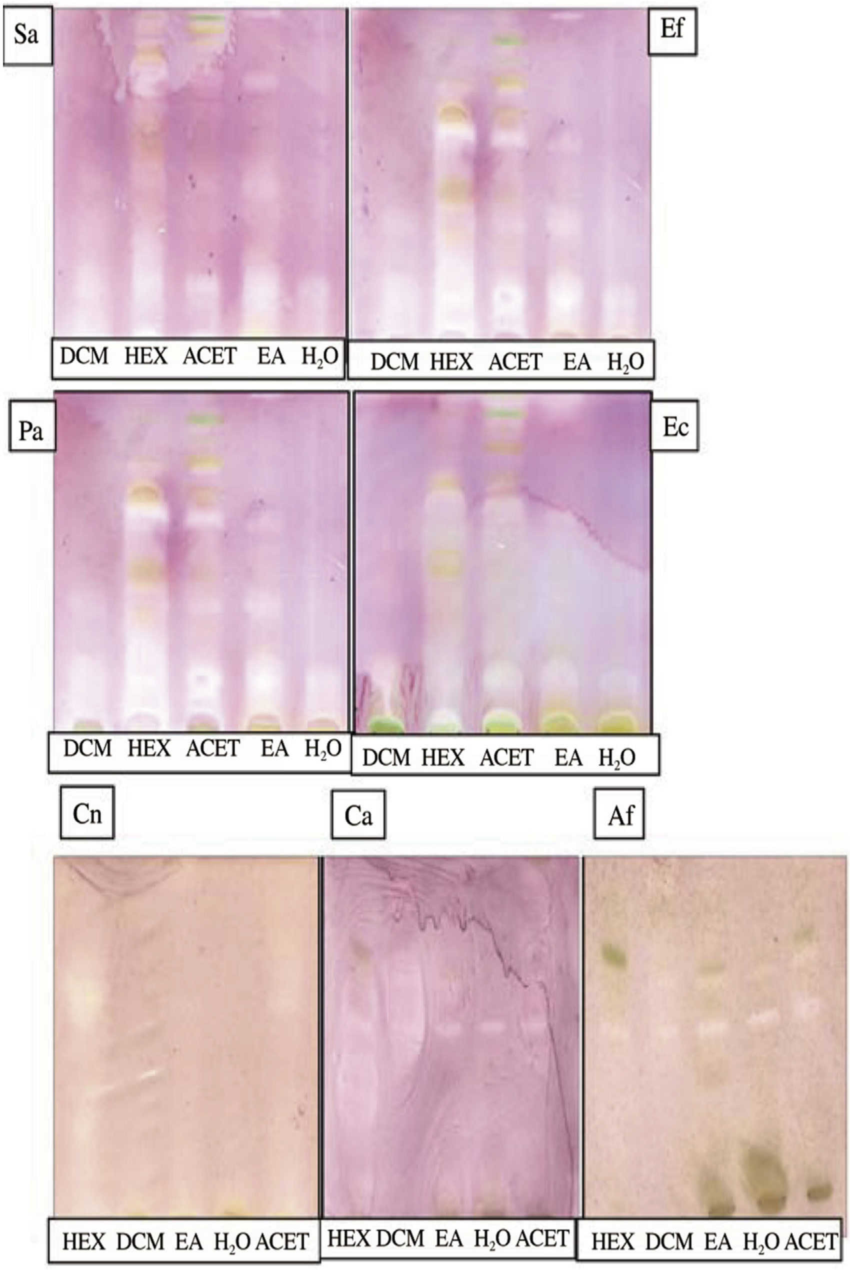

Figure 2. Bioautography of acetone extract and solvent fractions developed in HEX: EA (60:40) and sprayed with bacterial *S. aureus*, *E. faecalis*, *P. aeruginosa* and *E. coli* and fungal: *C. neoformans*, *C. albicans* and *A. fumigatus*.

Table 1

MIC (mg/mL) of crude acetone and solvent fractions against bacteria after 16 h incubation at 37 °C.

Organism	Sa positive	Ef positive	Ec negative	Pa negative
DCM	0.320 ± 0.080	0.630 ± 0.010	1.250 ± 0.180	0.320 ± 0.220
HEX	0.620 ± 0.030	1.250 ± 0.090	2.500 ± 0.260	0.320 ± 0.010
ACET	0.320 ± 0.090	0.800 ± 0.120	1.250 ± 0.040	0.620 ± 0.020
EA	0.320 ± 0.070	1.250 ± 0.100	0.080 ± 0.005	0.320 ± 0.090
H_2O	0.16 ± 0.008	0.320 ± 0.030	0.320 ± 0.040	0.160 ± 0.080
Negative control	>2.500 ± 0.180	>2.50 ± 0.180	>2.500 ± 0.180	>2.500 ± 0.180
Gentamicin (positive control)	0.040 ± 0.009	0.040 ± 0.003	0.320 ± 0.001	0.040 ± 0.007

Table 2

MIC values (mg/mL) of crude acetone extract and solvent fractions against fungal after 16 h incubation at 37 °C.

Organisms	Ca	Cn	Af
DCM	0.160 ± 0.020	0.080 ± 0.001	0.160 ± 0.050
HEX	0.320 ± 0.030	0.160 ± 0.002	0.080 ± 0.020
ACET	1.250 ± 0.090	0.320 ± 0.007	0.080 ± 0.030
EA	1.250 ± 0.120	0.080 ± 0.005	0.080 ± 0.040
H_2O	0.620 ± 0.015	0.080 ± 0.008	0.080 ± 0.010
Negative control	>2.500 ± 0.180	>2.500 ± 0.180	>2.500 ± 0.180
Amphotericin B (positive control)	0.020 ± 0.001	0.040 ± 0.008	0.040 ± 0.002

Table 3

Total activity (mL/mg) of crude acetone extract and solvent fractions of *C. erythrophyllum* leaves.

Extracts/organisms	Sa	Ef	Ec	Pa	Ca	Cn	Af	Average
ACET	93.75	24.00	93.75	93.75	24.00	93.75	375.00	114.00
DCM	17.70	8.98	4.53	17.69	35.38	70.75	35.38	27.20
Hex	1.07	0.53	0.26	2.06	2.06	4.13	8.25	2.62
EA	10.81	2.77	43.25	10.81	2.77	43.25	43.25	22.42
H_2O	21.75	10.88	10.88	21.75	5.61	43.50	43.50	22.55
Average	29.02	9.43	30.54	29.21	13.96	51.08	101.08	–

Table 4

Radical scavenging potentials (IC_{50}, mg/mL) of the *C. erythrophyllum* leaves extracts (n = 3, mean ± SD).

Extracts/organisms	DPPH	$ABTS^+$	Hydroxyl
ACET	0.131 ± 0.003	0.076 ± 0.020	0.015 ± 0.003
DCM	0.049 ± 0.007	0.256 ± 0.006	0.045 ± 0.032
Hex	0.432 ± 0.009	0.216 ± 0.050	0.048 ± 0.040
EA	0.043 ± 0.010	0.040 ± 0.010	0.017 ± 0.007
H_2O	0.058 ± 0.020	0.018 ± 0.006	0.026 ± 0.005
Ascorbic acid	0.030 ± 0.007	0.016 ± 0.022	0.013 ± 0.004

4. Discussion

Mass extracted from the powdered leaf material of *C. erythrophyllum* using different solvents was in accordance with the literature [19]. Dichloromethane fraction was found to be the best extractant, extracting the highest quantity of the plant material, more than any other solvents. The extractability of acetone, hexane, dichloromethane, ethyl acetate, water solvents were consistent with observations reported by Masoko *et al.* [20].

Bioautography of Merck TLC F254 chromatograms worked well with all the tested pathogens as opposed to the difficulties encountered by Martini and Eloff [21], in that the TLC chromatograms for the bioautography functioned well with *S. aureus*, but not effective with the other bacteria [21]. The crude and the hexane fraction showed more antimicrobial components whilst the water and dichloromethane showed less number of active compounds against the four tested pathogens. These organisms are known to cause infective endo-carditis which is a thoughtful complication of bacteremia [8,22]. Because the water extracts showed the least activity against the selected bacterial strains, it might suggest that traditional healers who normally use water as a solvent for their preparation could be missing out some of the active compounds that are present in the plant [22,23].

The clear zones on the chromatogram indicate the inhibition of growth by the plant extract. Hexane fraction has the highest number of antibacterial compounds in all organisms followed by crude extract then ethyl acetate fraction. Water and DCM fractions showed the poor activity of all organisms. Only a few compounds in the crude extract and other fractions inhibited the growth of antifungal microorganisms, in some cases, it was found that the compound responsible for the activity is the same due to similar Rf values. The results obtained in this assay showed that the leaf extracts of *C. erythrophyllum* possess good antibacterial activity.

In most part of the world, medicinal plant extracts have been reported to possess antibacterial, antifungal, and antiviral properties [24]. These potentials are indicators that the interaction between various phytochemicals, especially phenolic and flavonoid compounds in medicinal plant species are involved in reducing the risk of various deteriorating diseases [6,8,19]. Plant extracts with low MIC values could be a good source of bioactive compounds with antimicrobial strength. In this study, water fraction exhibited the highest activity against gram-positive bacteria: *S. aureus* (0.16 mg/mL), *E. faecalis* (0.32 mg/mL) and gram-negative: *P. aeruginosa* (0.16 mg/mL). The extracts demonstrated moderate inhibition against the studied bacteria apart from ethyl acetate fraction possessing strong inhibition against gram-negative: *E. coli* (0.08 mg/mL). Acetone extract showed high activity against *S. aureus* (0.32 mg/mL), dichloromethane extract against *S. aureus* and *P. aeruginosa* (0.32 mg/mL). Hexane extract against *P. aeruginosa* (0.32 mg/mL). The low activities of *C. erythrophyllum* extracts against the studied bacteria could be attributable to the strains high resistance, owing to the presence of an extra outer membrane in their cell wall acting as an obstacle for the extracts permeability [25]. From the results, water and ethyl acetate fractions were observed to be the best extractants for MIC assay, and this good activity might be due to materials such as soluble phenolic and polyphenolic compounds [26,27].

High MIC value was found in hexane fraction (2.5 mg/mL) against *E. coli*, while 1.25 mg/mL for hexane and dichloromethane fractions were observed against *E. coli* and *E. faecalis*. The ethyl acetate, dichloromethane and hexane fractions investigated also showed very interesting results as displayed by ethyl acetate with 0.08 mg/mL against the *E. coli*; gram-negative bacteria. The *in vitro* antibacterial activity of *C. erythrophyllum* extracts showed a substantial activity against gram-positive bacteria, while gram-negative bacteria specifically, *E. coli* exhibited higher resistance [28]. The MIC value found for water extract against *S. aureus* supports usage of water as a good extracting solvent for the traditional healers. *S. aureus* is known to cause infective endocarditis that is a severe complication of bacteremia [29].

The MIC values of the fungal assay followed the same order of the antibacterial assay. The highest MIC values were found for the crude acetone and ethyl acetate extracts against *C. albicans* (1.25 mg/mL) and dichloromethane fraction (0.16 mg/mL) against *C. albicans* and *A. fumigatus*. Crude extract and other fractions showed MIC value of 0.02 mg/mL against *C. neoformans*. Hexane, ethyl acetate and water fractions and crude extract also had the lowest MIC of 0.08 mg/mL against *A. fumigatus*.

In this study, the MIC of *C. erythrophyllum* extracts ranging from 0.08 to 1.25 mg/mL exhibited significant inhibition against the studied bacterial and fungal strains but lower activities compared to standard drugs: gentamicin and amphotericin B; antibacterial and antifungal agents respectively. Although plant extracts exhibiting MIC values ranging from 1.25 to 10 mg/mL possess high potent [30]. The results obtained for the potential activities of the leaf extracts against the tested pathogens agree with the results reported by Martini and Eloff for *Combretum* spp. with lowest minimum inhibitory concentration for *S. aureus* being 0.05 mg/mL [21]. It can be indicated from the results that *C. erythrophyllum* extracts possess good antibacterial and antifungal activity with respect to its activities against the studied pathogenic strains. The activity of the extracts can be related to their flavonoid and phenols content found effective as antimicrobial substances against a wide array of microorganisms studied *in vitro* [19,31].

Total activity of the extracts gives an indication of the efficacy to which the active constituents present in 1 g can be diluted and still inhibits the growth of the test organism. This value is calculated in relation to the MIC value of the extract [31]. To determine which extract is the most efficient as a source of antimicrobial compounds; the total activity of the extracts was calculated. The value of the total activity indicates the volume to which the biologically active compounds present in 1 g of dried plant extract can be diluted and still kill the bacteria. Extracts possessing higher total activity values in mL/mg (efficacy) are considered the best for isolation of potential bioactive compounds [32]. The acetone extract had the highest average total activity compared to other extracts with the average value of 114 mL/mg. For dichloromethane extracts, the average total activity was found to be 27.20 mL/mg followed by water fraction (22.55 mL/mg) while hexane extract exhibited the lowest potential with a total activity of 2.62 mL/mg. Consequently, it's interesting to know that 1 mg of *C. erythrophyllum* acetone and dichloromethane extracts can be diluted to 114 mL and 27.20 mL respectively, with water and still inhibit the growth of bacterial and fungal strains [32]. Hence, other extracts with moderate activity are worthy of investigation as studies have shown that there is synergy among different compounds within an extract [2,32].

Prevention of radical damage in the human systems, caused by free radicals due to oxidative reactions of biomolecules is very important using drugs that may be rich in antioxidants [33]. Oxidative stress symbolizes an inequity between the systemic appearance of reactive oxygen species (ROS) and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. In humans, oxidative stress is involved in the development of many diseases or sometimes it may intensify their symptoms. These include cancer [34], atherosclerosis, heart failure [35] and bipolar disorder [36]. The findings of this study revealed that solvent extracts of *C. erythrophyllum* exhibited medium to strong scavenging potentials against the evaluated radicals.

DPPH assay is one of the most widely used methods for screening antioxidant activity of plant extracts [37]. The degree of solution discoloration indicates the scavenging efficiency of the added substance (antioxidant). All the fractions and crude acetone extracts of *C. erythrophyllum* showed H-donor activity. The highest DPPH radical scavenging activity was shown by the ethyl acetate fraction [IC_{50} (0.043 ± 0.01) mg/mL], followed by dichloromethane [IC_{50} (0.049 ± 0.007) mg/mL], while the hexane fraction exhibited the lowest DPPH radical scavenging potential of IC_{50} (0.432 ± 0.009) mg/mL. These activities were found to be less when compared to that of the standard: ascorbic acid [IC_{50} (0.03 ± 0.007) mg/mL]. The DPPH radical scavenging ability of the extracts can be ranked: ethyl acetate > dichloromethane > water > acetone > hexane. The low antioxidant activity in the DPPH assay is associated with the quantity of the phenolic compounds in the fraction or an extract [38]. Consequentially, the synergism between the antioxidants in the extracts points to the fact that antioxidant action does not only depends on the concentration but however, depends on the structure and the interaction between the enclosed antioxidants [39]. Therefore, this suggests that there are less phenolic compounds in the hexane fraction as compared to the crude acetone extract and other three different fractions.

The extracts of *C. erythrophyllum* leaf were found to be fast and effective scavengers of the ABTS radical. Antioxidant activity of test extracts was determined by measuring the rate at which the $ABTS^{+}$ radical cation was decolourized as the percentage inhibition at an absorbance of 734 nm [40]. The $ABTS^{+}$ radical cation scavenging capacity of the extracts showed that the water residue fraction with IC_{50} = (0.018 ± 0.006) mg/mL possess the highest ABTS radical scavenging activity (IC_{50}) compared to the crude acetone extract [(0.076 ± 0.020) mg/mL], hexane fraction [(0.216 ± 0.050) mg/mL], ethyl acetate [(0.040 ± 0.010) mg/mL] and dichloromethane [(0.256 ± 0.006) mg/mL]. Compared to the reference, which was ascorbic acid [(0.016 ± 0.022) mg/mL], the tested fractions and acetone extract were found to be less potent. The activities of the test samples can rank: ascorbic acid > water fraction > ethyl acetate extract > acetone extract > hexane fraction > dichloromethane extract. The potential to scavenge $ABTS^{+}$ radical by the fractions and crude acetone extracts of *C. erythrophyllum* was found to be higher than that of DPPH radical. Stereoselectivity of the radicals or solubility of the extracts in different testing systems has been reported factors that might likely affect the capacity of extracts to react and quench different radicals [41].

Hydroxyl radicals are the most reactive and predominant radicals, amongst ROS that are endogenously generated *in vivo* through metabolism to initiate cell damage [42]. The effect (IC_{50}) of hexane, ethyl acetate and dichloromethane fractions, water residue and acetone extract on deoxyribose damage induced by Fe^{3+}/H_2O_2

in a concentration-dependent manner. The radicals emanating from the Fenton reaction combine with biomolecules found in living cells like nucleotides in DNA and bring about strand breakage leading to carcinogenesis, mutagenesis, and cytotoxicity [43,44]. Among the five extracts, marked scavenging effect was observed in the case of acetone crude extract [(IC_{50} = 0.015 ± 0.003) mg/mL] when compared to the scavenging activities of the hexane, ethyl acetate, dichloromethane and water fractions. The lowest scavenging activity was observed for dichloromethane [IC_{50} = (0.045 ± 0.032) mg/mL] and hexane [IC_{50} = (0.048 ± 0.040) mg/mL] extracts respectively, which can be ranked in the order: acetone > water > ethyl acetate > dichloromethane > hexane. Although the synthetic antioxidant (ascorbic acid) exhibited stronger hydroxyl scavenging activity (IC_{50} = 0.013 ± 0.004), comparing it to that of the plant extracts. However, it is evident that the extracts are effective scavengers of ROS particularly the hydroxyl radical and could serve as free radical scavengers.

The use of effective and safe plant extracts by traditional healers need to be encouraged since studies have confirmed the presence of an enormous bioactive compounds in the different parts of medicinal plants. These active compounds include steroids, tannins, alkaloids, terpenoids, phenols, saponins, and flavonoids exhibit several biological activities [15–19]. Toxicity testing is very important as some plants' extracts may be toxic; therefore safety testing is required [45]. In this study, toxicity testing of the crude acetone extract and the corresponding fractions were investigated for a better understanding of the characteristic cytotoxicity effect of *C. erythrophyllum* extracts on Vero monkey kidney cells.

The cytotoxic activity extracts of *C. erythrophyllum* against the Vero kidney cells by the MTT assay ranged from 34.80 to 223.10 μg/mL. Among the extracts, crude acetone had LC_{50} value of 34.80 μg/mL, dichloromethane 36.60 μg/mL, ethyl acetate 94.70 μg/mL, water 223.1 μg/mL while the standard drug (doxorubicin) gave LC_{50} of 7.19 ± 0.966 μg/mL. According to American National Cancer Institute (ANCI), the LC_{50} limit for consideration of a crude extract favorable for further refinement to isolate biological active compounds should be lower than 30 μg/mL [46]. Interestingly, the crude acetone and dichloromethane extracts showed a moderate cytotoxic activity of 34.80 and 36.60 μg/mL respectively. This agrees with the previous report on acetone extract of *Combretum roxburghii* (roxburgh) with moderate cytotoxic activity in live cell assay using Jurkat cells (tumor cell lines) [47]. The ethyl acetate extract of the leaves of *C. erythrophyllum* exhibited weak cytotoxic activity with LC_{50} value of 94.70 μg/mL while water fraction exhibited the lowest LC_{50} of 223.1 μg/mL against Vero kidney cells, hence toxic [48]. Cytotoxicity activity of cardamonin and pinocembrin isolated from *Combretum apiculatum* subsp. *apiculatum* leaves extracts were evaluated on Vero kidney cells using the MTT assay, but at concentrations higher than 50 μg/mL, the cells were not viable [49]. Although, the cytotoxicity of four kaempferol derivatives against Vero cells reported by Ibrahim *et al.* [50] was not observed up to a concentration of 100 μg/mL. *Elaeis guineensis* extract demonstrated the cytotoxicity indices as a measure of percentage cell mortality calculated by MTT assay in Vero cells with LC_{50} value 22 μg/mL [51]. The results of the extracts provide some indication for the traditional use of *C. erythrophyllum* leaves in treating infections, however, further investigation into the *in vitro* and *in vivo* cytotoxic activity against human cancer cell lines is required.

In conclusion, TLC chromatograms sprayed with vanillin indicated that the DCM and EA fractions extracted from *C. erythrophyllum* were efficient, indicating the presence of multiple medicinally active compounds. The data and observations from this study have demonstrated the dependence on ethnomedicinal information as a strategic tactic for harnessing ROS. Hence, the extracts of *C. erythrophyllum* were good sources of antioxidant for treating ailments associated with oxidative stress. The ethyl acetate fraction, water fraction, and crude acetone extract showed marked DPPH, $ABTS^+$ and hydroxyl radical scavenging abilities, and this might be due to the presence of significant amount of phenolic content and bioactive secondary metabolites. The minimum inhibitory concentration values proved that the leaf extracts possess potential to treat infections from bacterial and fungal pathogens. The mechanistic action from the assays justified the use of this plant for use in folk medicine in treating infections. Although, the weak cytotoxicity of ethyl acetate and water extracts indicates that the safety use of the plant could be of concern. Hence, the study recommends for future investigations on the active constituents of *C. erythrophyllum* for proper evaluation of the pharmacological properties. The possible improvement of the bioactive compounds as promising chemotherapeutic drugs via *in vitro* and *in vivo* anticancer activity against various human cancer cell lines.

Conflict of interest statement

The authors declare that they have no competing interests.

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