In vitro bioactivity of the fractions and isolated compound from *Combretum elaeagnoides* leaf extract against selected foodborne pathogens

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ABSTRACT

Ethnopharmacological relevance: *Combretum* species are used traditionally for the treatment of diarrhea, hookworm, fever, inflammation, pain and infectious diseases. Infections are commonly caused by the intake of food contaminated with foodborne pathogens. These are a significant concern in the food industry owing to their ability to form biofilms and cause food spoilage, despite the availability of modern food preservation techniques. *Combretum elaeagnoides* Klotzsch (Combretaceae) is used in southern African traditional medicine against infections and diarrhoea.

Aim of the Study: This study evaluated the antimicrobial ability of C. *elaeagnoides* leaf fractions and the isolated compound quercetin-3-*O*-rhamnoside against a panel of foodborne pathogens, and biofilms formed by them. The samples were also assessed for their antioxidant activity and cytotoxicity.

Materials and Methods: Fractions prepared from the methanol extract of the leaves, and a bioactive compound (quercetin-3-*O*-rhamnoside) isolated from the ethyl acetate fraction were investigated for activity against nine reference and clinical strains of foodborne pathogens. The microdilution method was used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the fractions and compound. The inhibition of biofilm formation and the crystal violet staining assays were used to determine the antibiofilm efficacy. The DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay and the 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) electron reduction assay were used to determine the antioxidant potential of the fractions and compound. The cytotoxicity was assessed using the 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay against Vero African monkey kidney cells.

Results: The fractions were active against all tested organisms, with MIC values ranging from 0.03-1.25 mg/mL. The best MBC was 0.63 mg/mL. All the fractions and the purified compound inhibited biofilm formation of *Staphylococcus aureus* and *Salmonella* Typhimurium, with percentage inhibition values greater than 50% at 1 mg/mL. The compound had very promising antibiofilm activity against *Escherichia coli* **1** (ATCC 25922) with percentage inhibition of >150%. The compound and fractions had good radical scavenging potential against the DPPH and ABTS radicals. Quercetin-3-*O*-rhamnoside and the fractions were relatively non-cytotoxic.

Conclusion: The ability of the fractions and compound to reduce and inhibit biofilm biomass and their promising antioxidant potential provide motivation to further investigate the use of plants to protect food products from contamination, as well as to treat infections characterised by bacterial biofilms.

Keywords: *Combretum elaeagnoides,* foodborne pathogens, antibiofilm, antioxidant, antibacterial, cytotoxicity.

List of abbreviations: ABF, antibiofilm activity; ABTS, 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid); ATCC, American Type Culture Collection; BuOH, butanol; CeB, *Combretum elaeagnoides* butanol fraction; CeH, *C. elaeagnoides* hexane fraction; CeD, *C. elaeagnoides* dichloromethane fraction; CeE, *C. elaeagnoides* ethyl acetate fraction; CeH_20, *C. elaeagnoides* water fraction, Ceo, quercetin-3-*0*-rhamnoside; CVS, crystal violet staining; DCM, dichloromethane; DMSO, dimethyl sulfoxide; DPPH, 2, 2-diphenyl-1-picrylhydrazyl; EPS, extracellular polymeric substance; EtOAc, ethyl acetate; Hex, hexane; IC_{50}, 50% inhibitory concentration; INT, *p*-iodonitrotetrazolium violet; LC_{50}, 50% lethal concentration;

MBC, minimum bactericidal concentration; MeOH, methanol; MEM, Minimal Essential Medium; MH, Mueller-Hinton; MIC, minimum inhibitory concentration; MTT, 3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide; NMR, Nuclear Magnetic Resonance; OD, optical density; TLC, thin layer chromatography; TSA, Tryptic Soy Agar; TSB, Tryptic Soy Broth

Compound studied: Quercetin-3-*O*-rhamnoside

1. Introduction

Medicinal plants have long been part of African and other cultures, used traditionally by the majority of the world population for treatment of various ailments. For example, in South Africa, it is estimated that 200 000 traditional healers are consulted by over 60% of the population, especially in rural areas (Van Wyk et al., 1997). Medicinal plant products have been utilised in the development of several new drugs due to the presence of different classes of bioactive compounds in the plant extracts.

Foodborne pathogens are a major source of concern in the food industry, largely due to their resistance to antimicrobial agents, which is enhanced by their ability to form biofilms. Microbial biofilms are of utmost importance to the food industry as a source of contamination leading to food spoilage or transmission of microbial diseases (Van Houdt and Michiels, 2010). It has been established that biofilm-forming microorganisms, including foodborne pathogens, are more resistant to antibiotics, food preservatives, disinfectants, biocides and other antimicrobial agents than their planktonic counterparts (Khiralla and El-Deeb, 2015).

There is a need for new and effective alternative antimicrobial and antibiofilm agents for controlling the growth and development of bacterial pathogens. Plants have an impressive ability to produce a myriad of bioactive secondary metabolites like flavonoids, tannins, alkaloids, glycosides, terpenoids, saponins, steroids, quinones and coumarins (Das et al., 2010). These bioactive molecules are the source of plant-derived antimicrobial substances and many have been reported to be highly efficient in treating bacterial infections (Srivastava et al., 2014, Fernebro, 2011).

Combretum species belong to the family Combretaceae and are native to Africa, Madagascar, Tropical Asia, and America with almost 300 species native to Southern Africa. Combretum *elaeagnoides* is particularly native to Botswana, Zimbabwe, Caprivi Strip, Mozambique and Zambia (Hyde et al., 2021; IPNI and WCSP, 2021). Many of the species are reputed to be used for the treatment of diarrhea, hookworm, fever, dysmenorrhea, infertility in women, leprosy, scorpion and snakebite, abdominal pains, backache, bilharzia, cough, syphilis, toothache and general body weakness (Hutchings et al., 1996, Mangoyi et al., 2012; Rogers and Verotta, 1996; McGaw et al., 2001). In addition, *Combretum* species are used in several cultures in folk medicine to treat microbial infections and inflammatory maladies such as headache, abdominal pains, cold, conjunctivitis and rectal prolapse. Other folk uses include as antidiuretics, antiseptic, lotions for eye infections as well as for toothache (Hutchings et al., 1996).

Several pharmacological activities of *Combretum* species and some of the isolated compounds have been reported from South Africa, Democratic Republic of Congo, Côte d'Ivoire, Burkina Faso, amongst others to have higher antimicrobial activities than currently used antibiotics in the market like chloramphenicol and ampicillin (Martini and Eloff, 1998; Eloff, 1999; McGaw *et al*., 2001; Atindehou *et al*., 2004 Masoko and Eloff, 2005; Eloff and McGaw, 2006; Gansané *et al*., 2010 Manga *et al*., 2012;). *Combretum elaeagnoides* Klotzsch leaves are used traditionally amongst the indigenous people of Zambia to treat diarrhoea, malaria tuberculosis, skin infections and sexually transmitted infections (STIs) (Chinsembu *et al*., 2016; 2019) and the species has also been reported to be used as an antifungal (Magwenzi et al., 2014). There is little to no information available on the antimicrobial, antibiofilm, antioxidant and cytotoxic activity of *C. elaeagnoides*, except for antifungal activity reported against *Candida* species (Mangoyi et al., 2012; Liu et al., 2007).

Following an increase in knowledge on their bioactivity, the use of plant phenolics and other active plant components as natural additives has gained much interest in the food and health industries, with studies highlighting the potential of these antioxidant and antimicrobial constituents as food preservatives, as well as functional food ingredients (Papuc et al., 2017; Takó et al., 2020). Previous studies have shown that antioxidants, which are mainly phenolics with high radical scavenging potential, also possess an array of biological activities such as antibacterial, antiviral and antitumour effects (Ferguson et al., 2004; Russo et al., 2004; Cushnie and Lamb, 2005; Song et al., 2005; Silva et al., 2009). In view of the above, this study

was designed to evaluate the biological potential of the fractions against selected foodborne pathogens and to isolate and identify bioactive secondary metabolites from *C. elaeagnoides*.

2. Materials and methods

2.1 Extraction, fractionation and thin layer chromatography(TLC) of *C. elaeagnoides* leaf extract

The plant material was collected in the Lowveld National Botanical Gardens (LNBG), Nelspruit, South Africa, in April 2017. The plant was identified and authenticated by Ms. Magda Nel and Ms. Elsa Van Wyk of the H.G.W.J. Schweickerdt Herbarium, Department of Plant and Soil Sciences, University of Pretoria, South Africa. An herbarium voucher specimen of *C. elaeagnoides* was prepared and deposited at the herbarium with a voucher specimen number PRU 123603.

The leaves were dried in a ventilated room for four weeks. The dried powdered leaves of *C. elaeagnoides* (462 g) were extracted in 4 620 mL of aqueous 80% methanol, soaked for 48 h and filtered through Whatman No 1 filter paper. The extraction process was repeated twice on the same plant material to fully extract the active components. The filtrate was concentrated using a rotary evaporator (Büchi, Germany) under reduced pressure at 40ºC. This afforded the crude extract of the plant material.

Solvent-solvent fractionation was deployed to partition the constituents of the crude extract based on polarity. The crude extract was suspended in distilled water and was successively partitioned in a separatory funnel with 300 mL each of hexane (Hex), dichloromethane (DCM), ethyl acetate (EtOAc) and butanol (BuOH) respectively. This yielded four solvent fractions along with the aqueous extract. The yield of each of the fractions was determined by applying the formula:

Yield = (weight of extract/ weight of dry sample) x 100

The fractions obtained were analyzed on Thin Layer Chromatography (TLC) plates where 10 µL of each fraction (of a 10 mg/mL aliquot) were loaded on TLC plates and developed using a mixture of chloroform:methanol ($CHCl_3$:MeOH, 4:1). The chromatograms obtained were sprayed with vanillin-sulphuric acid spray reagent for detection of chromogenic substances. The compound resulting from isolation and characterization of active plant constituents was also analyzed using TLC.

2.2 Isolation of bioactive compound from the ethyl acetate fraction of *C. elaeagnoides* leaf extract

2.2.1 Column chromatography

The ethyl acetate fraction was one of the most abundant fractions with promising antibacterial activity, and was selected for further fractionation and isolation of its bioactive constituents. Column chromatography was utilized to separate the compounds using silica gel (230-400 mesh, Merck) as stationary phase. The ethyl acetate fraction (27.0 g) was dissolved in methanol and adsorbed on silica gel (40.0 g). The methanol in the mixture was evaporated completely using a rotavapor. A glass column (100 x 2.5 cm) was rinsed several times with 100% hexane. Cotton wool was inserted in the bottom of the column using a glass rod, after which it was packed with a slurry of silica gel (270 g). Hexane (100 mL) was added and allowed to flow out to enable the silica gel to settle. A mixture of hexane and ethyl acetate (7:3) was poured into the column and the ethyl acetate fraction adsorbed on silica gel was carefully loaded onto the column. The polarity of the eluting solvent mixture was increased gradually from 70% hexane in ethyl acetate by increasing the volume of ethyl acetate by 10% up to 100%. The solvent mixture was then changed to a mixture of ethyl acetate and methanol starting with 10% MeOH up to 100% methanol. A total of 89 test tubes (25 mL each) were collected.

2.2.2 Bulking of fractions

Test tube fractions collected were developed on TLC plates using chloroform and methanol (9:1) as eluent. The chromatograms obtained were sprayed with vanillin in sulphuric acid. Fractions with similar TLC profiles were combined to give three subfractions: subfraction 1, subfraction 2 and subfraction 3. Subfraction 1 yielded a distinct spot on the TLC plate and was selected for purification and isolation of this major constituent.

2.2.3 Purification of subfraction 1 on Sephadex LH-20 column

A glass column (35 cm x 2 cm) packed with pre-soaked Sephadex LH-20 was flushed several times with 100% methanol to clean the column. A solvent mixture of chloroform:methanol (4:1) was prepared and 50 mL of the solvent mixture was added to the column while 10 mL was added to the sample (2.29 g). The sample was then dissolved using a sonicator. The sample was then applied onto the column and eluted with chloroform/methanol. The solvent mixture gradient was increased to 3:2; 2:3; 1:4 and finally to 100% methanol. Eluent was collected in 25 mL test tubes and analysed on TLC plates using DCM:MeOH (1:4) as the separating solvent. The test tubes numbered 15-20 showed a single spot on TLC analysis and were

combined following the similarity of their TLC profile. The solvent in the combined test tubes was allowed to dry under a stream of cold air to yield the compound (Ceo, 259.7 mg).

2.2.4 Spectroscopic characterization of the isolated compound

2.2.4.1 Characterization of compound (Ceo)

Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR) data were obtained using a 400 MHz Bruker spectrometer at the Department of Chemistry, University of Pretoria, South Africa.

2.3 Antibacterial activity

2.3.1 Source and quantification of bacterial strains

American Type Culture Collection (ATCC) bacterial strains (Gram-positive and Gram-negative) and clinical bacterial strains obtained from the Phytomedicine Research Laboratory in the Department of Paraclinical Sciences, Faculty of Veterinary Sciences University of Pretoria were used in this study. The bacterial strains used included: *Salmonella enterica* subsp. *enterica* serotype Typhimurium (*Salmonella* Typhimurium) ATCC 39183, *Salmonella* Enteritidis ATCC 13076, *Escherichia coli* **1** ATCC 25922, *Escherichia coli* **2** (clinical isolate), *Staphylococcus aureus* ATCC 29213, *Campylobacter jejuni* ATCC 33560, *Stenotrophomonas maltophilia* (clinical isolate), *Klebsiella pneumoniae* (clinical isolate) and *Enterobacter cloacae* (clinical isolate).

The bacterial strains were maintained on Mueller-Hinton (MH) agar (Fluka, Spain) at 4ºC. The bacterial inoculum was prepared and cultured in MH broth for 12-16 h at 37ºC. MH agar and broth were used for the minimum inhibitory concentration tests, while Tryptic Soy Agar (TSA) and Tryptic Soy Broth (TSB) were used in the minimum bactericidal concentration and biofilm assays.

2.3.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the fractions and compound isolated from *C. elaeagnoides*

The MIC of the fractions and isolated compound was determined following the method of Eloff (1998). The fractions were tested at a starting concentration of 2.5 mg/mL (from a stock concentration of 10 mg/mL) and the compound at a starting concentration of 2 mg/mL in 96-well microtitre plates and serially diluted two-fold to 0.02 mg/mL and 0.016 mg/mL respectively. Following this, 100 µL of the diluted overnight bacterial culture standardized in comparison to a McFarland standard No 1 were added to each

well. The positive control, gentamicin (0.1 mg/mL starting concentration), was used as the reference drug for the assay. The microtitre plates were covered with lids, sealed with parafilm and incubated overnight for at least 18 h at 37°C. As an indicator of bacterial growth, 40 µL of *p*-iodonitrotetrazolium violet (INT) (Sigma 0.2 mg/mL) dissolved in distilled water was added to the wells and incubated at 37°C for 1 h. The MIC values were recorded as the lowest concentration of the extract that inhibited bacterial growth, as indicated by a marked reduction in colour formation. The *p*-iodonitrotetrazolium violet turns to a red-pink formazan where bacterial growth is not inhibited. The assays were repeated three times in triplicate.

The MBC was determined by adding aliquots of 50 µL from the wells of the concentrations where there was no bacterial growth after incubation during MIC assays, to 150 µL of freshly prepared TSB. The preparations were thereafter spread on a TSA agar plate. These preparations were incubated at 37°C for 24 h. The lowest concentration of extract with no bacterial growth was recorded as the MBC value (Cohen et al., 1998).

2.4 Determination of anti-biofilm activity

2.4.1 Inhibition of biofilm biomass formation

The inhibition of biofilm biomass formation was assessed using the modified protocol previously described by O'Toole and Kotler (1998), Mohsenipour and Hassanshahian (2015) and Sandasi et al. (2010). Briefly, biofilm was allowed to form for 24 h to allow irreversible attachment. Biofilm production was achieved by aliquoting 100 µL of the respective culture (OD_{590} = 0.02 or 1.0 x 10^6 CFU/mL) into a sterile flat bottom 96-well microtitre plate and sterile sealed with a sealing tape. One hundred microliters (100 µL) of the sample (at a final concentration of 1 mg/mL from a stock of 2 mg/mL) and the respective controls were transferred to the wells of the sterile plate and incubated for 24 h at 37ºC without shaking. Appropriate control wells were included in the plate, including negative control (culture + media (TSB)), positive control (culture + TSB + antibiotic), sample control (sample + TSB), antibiotic control (antibiotic + TSB) and media control (TSB) for each test batch. After incubation, the modified crystal violet staining (CVS) assay (Sandasi et al., 2010) was performed to assess the biofilm biomass or adhered cell biomass.

In the CVS assay, the wells were carefully emptied and washed three times with sterile distilled water to remove any unattached cells. The plates were then air-dried and 150 µL of 96% methanol added to the wells for 15-20 min to fix the adherent cells. The plates were emptied, and the adhered cells stained with

100 µL of 0.1% crystal violet solution for 20 min at room temperature while covered with lids. The plates were washed five times with running tap water to remove any excess or unabsorbed stain. Thereafter, the biofilm biomass was assessed semi-quantitatively by re-solubilizing the crystal violet stain bound to the adherent cells with 150 µL of 100% ethanol. The absorbance of the plates was read at 590 nm using a microplate reader (Epoch™ Microplate Spectrophotometer). The mean absorbance (OD_{590nm}) of the sample was determined and results expressed as percentage inhibition using the equation below.

$$\% \text{ Inhibition} = \frac{(OD_{negative\ control} - OD_{media\ control}) - (OD_{sample} - OD_{sample\ control})}{(OD_{negative\ control} - OD_{media\ control})} \times 100$$

2.5 Antioxidant activity

2.5.1 Assay for free radical scavenging (DPPH) activity

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was done according to Gyamfi et al. (1999) with slight modifications. Initially, the optical density (OD) of the DPPH solution was adjusted to read between 0.9 and 1.00 at 517 nm. Thereafter, 160 µL of the DPPH solution was added to 40 µL of the different fractions and isolated compound at different concentrations (3.125–400 µg/mL). A similar amount of DPPH was also added to ascorbic acid and trolox (40 µL) which were the positive controls. The mixture was incubated in the dark for 30 min, and the absorbance measured at 517 nm using a microplate reader (Epoch, Biotek). A low absorbance of the solution indicated higher free radical scavenging activity. The percentage of scavenging activity was calculated using the formula below:

$$\% \text{ scavenging activity} = [(A0 - As)/A0] \times 100$$

Where A0 is absorbance of control (DPPH solution without sample), while As is absorbance of tested sample (DPPH plus sample).

The IC_{50} (50% inhibitory concentration) values of the fractions and isolated compound were determined using linear and non-linear regression curves, where appropriate, of percentages of scavenging activity against the actual or logarithm of concentrations. Each test was done in triplicate and results are presented as mean± standard error of mean (SEM).

2.5.2 The 2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

The stock solution was prepared by mixing 7 mM of ABTS with 2.45 mM of potassium persulfate and left at room temperature in the dark for 12–16 h (Re et al., 1999). The working solution was obtained by

diluting the stock solution to obtain an optical density reading of 0.70 ± 0.02 at 734 nm. One hundred and sixty microliters (160 µL) of the ABTS working solution was mixed with the samples (40 µL) at different concentrations and the absorbance measured after 7 min at 734 nm using a microplate reader (Epoch, BioTek). The percentage scavenging activity was calculated using this formula:

% scavenging activity= {(AO- AS)/AO} X 100

Where A0 is absorbance of control (ABTS solution without sample), while As is absorbance of tested sample (ABTS plus sample).

The IC_{50} (50% inhibitory concentration) values of the fractions and isolated compound were determined using linear and non-linear regression curves, where appropriate, of percentages of scavenging activity against the actual or logarithm of concentrations. Ascorbic acid and trolox were used as positive controls. Each test was done in triplicate and results were presented as mean± standard error of mean (SEM).

2.6 Cytotoxicity

2.6.1. Tetrazolium-based colorimetric assay (MTT)

The MTT (3-(4, 5- dimethylthiazolyl-2)-2.5-diphenyltetrazolium bromide) reduction assay was used to measure the cytotoxicity of C. *elaeagnoides* leaf fractions and the isolated compound quercetin-3-*O*-rhamnoside. The viable cell growth after incubation with test compounds was determined using the tetrazolium-based colorimetric assay (MTT assay) (Mosmann 1983) with some modification (McGaw et al., 2007). MTT is a yellow soluble dye that is reduced by live, but not dead cells to a purple formazan product that is insoluble in aqueous solutions The intensity of colour (measured spectrophotometrically) of the MTT formazan produced by metabolically active cells is proportional to the number of live cells present (Mosmann, 1983). The fractions and isolated compound were tested for cytotoxicity against Vero African green monkey kidney cells. The cells of a subconfluent culture were harvested and centrifuged at 200 × g for 5 min and re-suspended in growth medium. Minimal Essential Medium (MEM, Whitehead Scientific) supplemented with 0.1% gentamicin and 5% foetal calf serum (Gibco, Sigma-Aldrich) was used as growth medium. Cells were counted and a total of 10 000 cells seeded per well and adjusted to 100 µL with medium in columns 2 to 12 of a sterile 96-well microtitre plate (Nest, Whitehead Scientific). A 100 µL aliquot of medium was added to wells of column 1. The plates were then incubated for 24 h at 37°C in a 5% CO_2 incubator, until the cells were in an exponential phase of growth. Then MEM was aspirated from the wells and replaced with 200 µL of test compounds at differing concentrations (0.0075

to 0.1 mg/mL). Each dilution was tested in triplicate. The microtitre plates were incubated at 37°C in a 5% CO_2 incubator for 48 h with test fractions and compound. Untreated cells and positive control (doxorubicin chloride, Pfizer laboratories) were included.

After incubation, the MEM containing test substance was removed, and the cells washed with phosphate buffered saline (PBS) and fresh MEM (200 µL) added to each well. Then, 30 µL of MTT (5 mg/mL in PBS) was added to each well and the plates re-incubated for another 4 h at 37°C. After removing the MEM and MTT, the MTT formazan crystals was dissolved by adding 50 µL of dimethylsulfoxide (DMSO) to each well. The plates were shaken gently for about 10 min until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by reading the absorbance using a multimode microplate reader (Biotek Synergy, USA) at a wavelength of 570 nm with a corresponding reference wavelength of 630 nm. The wells in columns 1, containing medium and MTT but no cells, were used to blank the plate reader. The LC_{50} values was calculated as the concentration of test compound resulting in a 50% reduction of absorbance (which implies killing 50% of the cells) compared to untreated cells (using linear regression). The experiment was done three different times. The selectivity index was calculated as the ratio of cytotoxicity of the extracts to the antimicrobial MIC value (Shai et al., 2008).

2.7 Statistical analysis

The mean and standard error of means or mean and standard deviations where appropriate of the different assays were determined. The IC_{50} and LC_{50} were determined using linear and non-linear regression curves where necessary. One Way ANOVA was done to determine the means of the different groups with LSD and Duncan's multiple range test to ascertain the differences in the different groups of means using SPSS 26.0 computer software package. Differences at $P \leq 0.05$ were considered statistically significant.

3 Results and discussion

3.1 Percentage yield of fractions and isolated compound

The mass and percentage yield of the initial five fractions and bulked fractions are shown in Table 1. Of the fractions, the ethyl acetate fraction had the highest yield of 27.10 g while dichloromethane recorded the lowest yield of 3.98 g. The compound comprised 0.26 g and was obtained as a greenish-yellow to yellow coloured solid.

Table 1. Percentage yield of the different fractions and compounds

Fractions and compounds	Yield in gram (g)	Yield in percentage (%)
Butanol fraction	8.10	9.54
Hexane fraction	6.55	7.71
Dichloromethane fraction	3.98	4.69
Ethyl acetate fraction	27.10	31.91
Water fraction	10.32	12.15
Compound	0.26	0.31

3.2 Thin layer chromatography

The analyzed fractions and purified compound on thin layer chromatography (TLC) plates are shown in Figure 1.

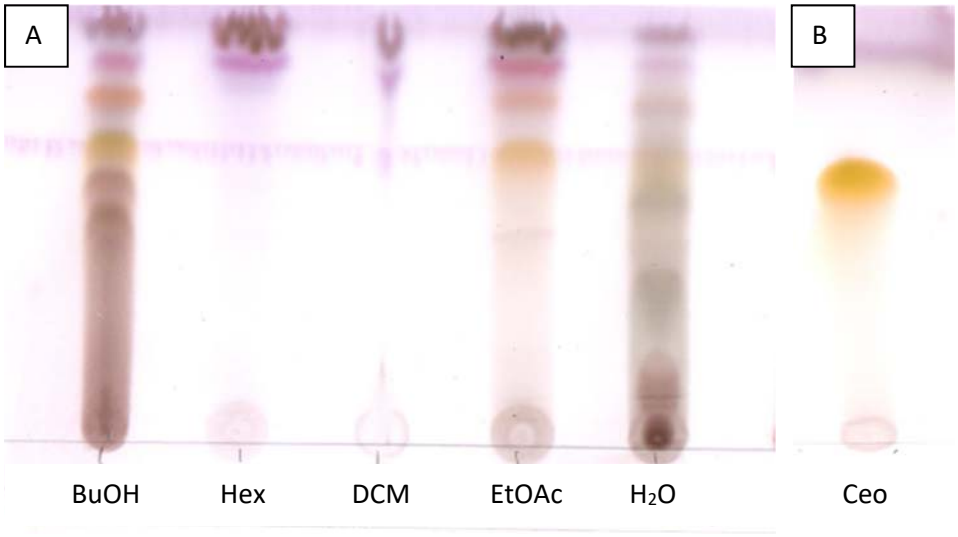


Figure 1. Thin Layer Chromatogram (TLC) of the fractions (A) and pure compound (B) derived from the aqueous methanol extract of *Combretum elaeagnoides* leaf extract. BuOH, butanol; Hex, hexane; DCM, dichloromethane; EtOAc, ethyl acetate; H_2O, water; Ceo, pure compound

3.3 Characterization of isolated compound

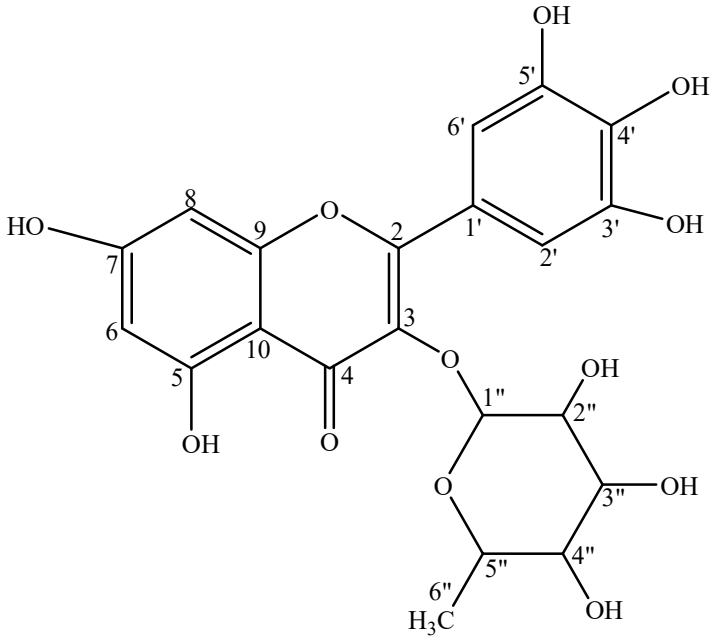
The ^1H NMR (DMSO-d_6) spectrum of the isolated compound showed a splitting pattern of a flavonol glycoside. There was a signal at δ 12.64 ppm that indicated the presence of a chelated 5-OH. The

substitution pattern of ring B was established from the multiplicity of its proton's signals. There was a doublet signal at δ 7.30 ppm (1H, H-2′, J= 2.4 Hz) indicating a meta orientation aromatic proton. A doublet of doublet signal appeared at δ 7.26 ppm (1H, H-6′, J =8.4 and 2.4 Hz) indicating a doubly coupled (meta and ortho related) aromatic proton. Another ortho coupled aromatic proton signal appeared at δ 6.88 ppm (1H, H-5′, 8.0 Hz). This substitution pattern revealed an ABX pattern for the ring B of the flavonol. The spectrum also showed the presence of two meta related ring A protons at δ 6.40 ppm (1H, H-8, 2.0 Hz) and δ 6.21 ppm (1H, H-6, J= 2.0 Hz). The presence of a sugar moiety in the flavonol was evident from the presence of the following signals in the spectrum: anomeric sugar signal at δ 5.25 ppm (1H, d, J= 1.2 Hz, anomeric H-1″), δ 4.97-3.20 ppm (H-2″- H-5″ rhamnosyl Hs) and δ 0.82 ppm, (3H, d, J= 6.0 Hz, rhamnosyl CH_3-6″). The ^{13}C NMR data of this compound is presented in Table 2. The isolated compound (Ceo) was identified as quercetin-3-*O*-rhamnoside (Figure 2) from the spectral data, which were in good agreement with the literature (Harborne and Mabry, 1982).

Previous work by Osborne and Pegel (1984, 1985) reported four compounds to have been isolated from the leaves of *C. elaeagnoides*. The previously isolated compounds, which were triterpenoids, include jessic acid (1 α, *β*-dihydroxyl), its α-L-arabinopyranoside, its methyl ester (methyl jessate) and methyl jessate 1α,11 α -oxide or methyl l α ,11 α -epoxy-3 *β* hydroxy-23-oxo-24-methylenecycloartan-30-oate. Following a literature search, the isolated compound quercetin-3-*O*-rhamnoside is herewith reported for the first time from the leaf extract of *C. elaeagnoides.* Previous reports show that the compound quercetin-3-*O*-rhamnoside has been isolated from *Combretum apiculatum* subsp. *apiculatum* (Aderogba et al., 2012), *Croton penduliflorus* (Aderogba et al., 2013a), *Croton menyharthii* (Aderogba et al., 2013b)*, Anacardium occidentale* (Ajileye et al., 2015) and *Euphorbia hirta* (Gopi et al., 2016).

Table 2. The ^{13}C NMR spectral data of the compound isolated from *C. elaeagnoides*

Carbon	Compound (DMSO-d_6)	Quercetin-3-*O*-rhamnoside (DMSO-d_6), (Harborne and Mabry, 1982)
2	157.5	156.7
3	134.3	134.6
4	177.9	178.0
5	161.4	161.6
6	98.8	99.0
7	164.3	164.4
8	93.8	93.9
9	156.6	157.5
10	104.2	104.5
1′	121.3	121.4
2′	115.8	115.9
3′	145.3	145.4
4′	148.6	148.7
5′	115.6	116.1
6′	120.9	121.2
1″	101.9	102.2
2″	70.5	70.4
3″	70.2	70.4
4″	71.3	71.7
5″	70.8	70.8
6″	17.6	17.8



Quercetin-3-*O*-rhamnoside

Figure 2. Structure of the isolated compound (quercetin-3-*O*-rhamnoside)

3.4 Minimum inhibitory concentration and minimum bactericidal concentration activities of fractions and isolated compound from *C. elaeagnoides*

The fractions (butanol, hexane, ethyl acetate, dichloromethane and water) of *C. elaeagnoides* had excellent to good antibacterial activity (Table 3). The identified compound (quercetin-3-*O*-rhamnoside) generally did not have strong antibacterial activity. The MIC results indicated that the more polar fractions (ethyl acetate and butanol) had the strongest growth inhibitory activity against the foodborne pathogens (Table 3). The ethyl acetate fraction generally had the best antibacterial activity against most of the test organisms. The dichloromethane (DCM) fraction at an MIC of 0.03 mg/mL had the best antibacterial activity against *E. coli* **1**. The hexane and ethyl acetate fractions showed the same inhibitory action (MIC = 0.63 mg/mL) against *E. coli* **2** (clinical strain) while the DCM and ethyl acetate fractions exhibited the same inhibitory action (MIC = 0.1 mg/mL) against *S. aureus.* The effect (MIC values) of the hexane, ethyl acetate and dichloromethane fractions of the plant compared to the control (gentamicin) against *E coli* 1 was not statistically different ($P \geq 0.05$). Similarly, the isolated compound (Ceo), dichloromethane and the water fraction showed no statistical difference ($P \geq 0.05$) in effect against *E. coli* 2. The fractions and compound

displayed statistically significant ($P \leq 0.05$) antibacterial activity compared to the control against *E. coli* 2, *K. pneumoniae* and *E. cloacae* (Table 3).

The MBC results revealed that the DCM fraction had the best bactericidal activity against *E. coli* **1** (0.31 mg/mL) followed by butanol and ethyl acetate fractions with MBC value of 0.63 mg/mL against the same organism. Dichloromethane and ethyl acetate fractions also had the same MBC value of 0.63 mg/mL against *S. aureus* (Table 3). It is interesting to note that *E. coli* **1** was the most susceptible organism while *E. coli* **2** and *E. cloacae* were the most resistant to the fractions and compound from *C. elaeagnoides*. It is however not surprising that the clinical isolate of *E. coli* was more resistant than the reference ATCC strain.

Table 3. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the fractions and isolated compound from *C. elaeagnoides* leaf extract

Test Organisms	Fractions and compound from *C. elaeagnoides* MIC (mg/ml)													
	CeB		CeH		CeD		CeE		CeH_20		Ceo		Control	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli **1**	0.26^{a}±0.05	0.63	0.09^{b}±0.04	0.31	0.03^{b}±0.01	0.08	0.06^{b}±0.00	0.16	0.52^{c}±0.11	2.5	2.5^{d}±0.00	Na	0.02^{b}±0.00	0.02
E. coli **2**	1.25^{a}±0.00	>2.5	0.63^{b}±0.00	2.5	2.5^{c}±0.00	Na	0.63^{b}±0.00	2.5	2.5^{c}±0.00	Na	2.5^{c}±0.00	Na	0.02^{d}±0.00	0.02
S. aureus	0.31^{a}±0.00	1.25	0.63^{b}±0.00	2.5	0.1^{a}±0.04	0.63	0.1^{a}±0.04	0.63	$0.8^{b}4$±0.21	2.5	2.5^{c}±0.00	Na	0.12^{a}±0.04	0.12
S. Typhimurium	0.63^{a}±0.00	>2.5	2.5^{b}±0.00	Na	0.31^{c}±0.00	1.25	0.63^{a}±0.00	2.5	0.52^{c}±0.11	2.5	1.25^{d}±0.00	>2.5	0.42^{c}±0.11	0.42
S. Enteriditis	1.04^{a}±0.21	>2.5	0.63^{bc}±0.00	1.25	0.37^{cd}±0.16	1.25	0.63^{bc}±0.00	2.5	0.84^{ab}±0.21	>2.5	2.5^{e}±0.00	Na	0.03^{d}±0.01	0.03
C. jejuni	0.73^{b}±0.21	>2.5	0.42^{bc}±0.11	2.5	0.21^{c}±0.05	1.25	0.42^{bc}±0.11	1.25	2.5^{a}±0.00	Na	2.5^{a}±0.00	Na	0.09^{c}±0.04	0.09
S. maltophilia	0.63^{c}±0.00	2.5	0.63^{c}±0.00	>2.5	1.25^{b}±0.00	>2.5	0.42^{cd}±0.11	2.5	1.67^{b}±0.42	>2.5	2.5^{a}±0.00	Na	0.02^{d}±0.00	0.02
K. pneumoniae	0.37^{a}±0.11	2.5	0.63^{b}±0.00	2.5	0.63^{b}±0.00	2.5	0.31^{a}±0.00	2.5	0.63^{b}±0.00	2.5	2.5^{c}±0.00	Na	0.02^{d}±0.00	0.02
E. cloacae	1.25^{a}±0.00	>2.5	0.63^{b}±0.00	2.5	2.5^{c}±0.00	Na	0.42^{d}±0.11	2.5	1.25^{a}±0.00	>2.5	2.5^{c}±0.00	Na	0.02^{e}±0.00	0.02

CeB (*Combretum elaeagnoides* butanol fraction), CeH (*C. elaeagnoides* hexane fraction), CeD (*C. elaeagnoides* dichloromethane fraction), CeE (*C. elaeagnoides* ethyl acetate fraction), CeH_20 (*C. elaeagnoides* water fraction), Ceo (quercetin-3-*O* rhamnoside), Control (Gentamicin), Na (Not applicable as MIC values were above the highest concentrations tested). Across the rows, values with one or two similar alphabetical letters are statistically non-significant ($P \geq 0.05$) while values with one or two different alphabetical letters are significantly different ($P \leq 0.05$).

3.5 The antibiofilm potential of the fractions and isolated compound from *C. elaeagnoides*

The fractions and isolated compound had varying effects on the growth and development of biofilms by the foodborne pathogens. Some of the fractions and the isolated compound had profound antibiofilm activity (ABF) against some of the organisms while others enhanced biofilm growth, expressed as 0% inhibition. Two of the fractions (hexane and butanol) had strong ABF activity against *K. pneumoniae* with percentage inhibition greater than 200 percent (>200%). Interestingly, none of the fractions but only the compound had ABF activity against *E. coli* **1** (ATCC 25922). This result is dissimilar to the report by Sandasi et al. (2010) where the ATCC isolate of *Listeria monocytogenes* was more sensitive than the clinical isolate to the tested samples.

Surprisingly, the planktonic cells of *E. coli* **1** (ATCC 25922) were sensitive to the fractions and compound in the antimicrobial studies (MIC and MBC). The isolated compound (Ceo) had excellent ABF activity with percentage inhibition value of >150% against *E. coli* **1**. It is worthy to note that *S. aureus* and *S.* Typhimurium were the most sensitive organisms as all the fractions and isolated compound had good antibiofilm activity with percentage inhibition values of >50% (Table 6). These results show that the inhibition of biofilm growth and development was more difficult to achieve than inhibiting growth of planktonic cells of the organism. This was also observed by Sandasi and colleagues in their investigation of the ABF activity of selected herbs, spices and beverages against *Listeria monocytogenes* (Sandasi et al., 2010).

A factor that has been linked to the ability of foodborne pathogens to form biofilms and express resistance is the presence of an EPS (extracellular polymeric substance, or glycocalyx) that encompasses the bacteria, as well as the negative charge on the EPS that limits the infiltration of molecules by charge attraction, thereby conferring resistance properties to the biofilm (Denyer et al. 2008). Another route through which these pathogens form resistant biofilms includes deactivation of the antimicrobial agent and efflux pumps that expel drugs from the cells (Denyer et al. 2008). Also, the slow growth rate in biofilms compared to free floating cells following reduced nutrient and oxygen supply has been reported as another factor (Costerton et al. 1999; Mah and O'Toole 2001).

It is interesting to note that some of the samples in this study enhanced biofilm formation. This observation could be as a result of the samples containing compounds with the capacity to produce conditioning films

for microbial adhesion (Sandasi et al., 2010). This enhancement in the growth and development of biofilm correlates with earlier studies that certain metabolites stimulate the growth of microorganisms (Ofek et al., 2003; Sandasi et al., 2008). Additionally, some components of essential oils were reported to stimulate the growth and development of preformed *L. monocytogenes* biofilm (Sandasi et al., 2008). Ofek and co-workers showed that plant lectins may improve the adsorption of cells onto a surface by acting as receptors of bacterial glycan, thus promoting cell attachment (Ofek et al., 2003). The enhanced attachment found upon exposure to some of the tested samples can be attributed to the presence of metabolites that support the development of the biofilms.

Table 4. Effect of fractions and isolated compound from *C. elaeagnoides* leaf extract on the growth and development of biofilm of selected foodborne pathogens

Test organisms	**Percentage inhibition of biofilm (%)**					
	CeB	**CeH**	**CeD**	**CeE**	**CeH20**	**Ceo**
E. coli 1	0.00*	0.00*	0.00*	0.00*	0.00*	153.77
E. coli 2	62.52	119.16	0.00*	0.00*	0.00*	269.78
S. aureus	77.39	66.69	77.46	71.93	80.57	67.59
S. Typhimurium	74.01	70.37	52.35	88.02	73.62	69.75
S. Enteritidis	0.00*	0.00*	0.00*	0.00*	63.35	0.00*
C. jejuni	67.24	0.00*	0.00*	52.27	0.00*	119.36
S. maltophilia	0.00*	0.00*	76.15	0.00*	0.00*	0.00*
K. pneumoniae	247.76	237.51	0.00*	91.53	54.17	0.00*
E. cloacae	118.30	0.00*	0.00*	0.00*	66.40	0.00*

***= fractions and compound that enhance biofilm growth (negative inhibition),** CeB (*Combretum elaeagnoides* butanol fraction), CeH (*C. elaeagnoides* hexane fraction), CeD (*C. elaeagnoides* dichloromethane fraction), CeE (*C. elaeagnoides* ethyl acetate fraction), CeH_20 (*C. elaeagnoides* water fraction), Ceo (quercetin-3-*O* rhamnoside)

3.6 Antioxidant evaluation

Antioxidants have the ability to neutralize or destroy reactive oxygen species or free radical chain of reaction before they cause damage to the cell (Gupta, 2015; Hakiman and Maziah 2009). The DPPH and ABTS non-enzymatic antioxidant assays were conducted. The antioxidant potential of the fractions and

isolated compound from *C. elaeagnoides* expressed as IC_{50} against the DPPH and ABTS radicals is presented in Table 5. In this study, the fractions and isolated compound (Ceo) had good to excellent antioxidant activity against the DPPH and ABTS radicals with IC_{50} values ranging from 0.01 to 7.00 and 0.01 to 20.00 µg/mL respectively, compared to the positive controls (vitamin C and trolox) with IC_{50} values of 0.39 to 2.64 µg/mL for DPPH and 0.36 to 0. 74 µg/mL for ABTS respectively. The water fraction with an IC_{50} value of 0.06 µg/mL had the best DPPH radical scavenging activity amongst the fractions but was less active than the compound (IC_{50} = 0.01 µg/mL). However, the DPPH scavenging effect of the water fraction was statistically not different ($P \geq 0.05$) from the butanol and hexane fractions and the pure compound when compared to Vitamin C (control) (Table 5). The fractions, except for the ethyl acetate fraction, were more active against the DPPH radical than the two positive controls (trolox, IC_{50} = 2.64 µg/mL and vitamin C, IC_{50} = 0.39 µg/mL). The fractions and the isolated compound had statistically significant ($P \leq 0.05$) scavenging effect when compared to trolox (control). The butanol fraction was the most active fraction against the ABTS radical with IC_{50} value of 0.01 µg/mL. The effect of the butanol fraction was statistically significantly different ($P \leq 0.05$) from the other fractions, the isolated compound and trolox except vitamin C (Table 5).

The compound quercetin-3-*0*- rhamnoside had the best antioxidant activity against the DPPH and ABTS radicals with IC_{50} = 0.01 and 0.90 µg/mL respectively, compared to the fractions and positive controls (vitamin C and trolox) (Table 5). Quercetin-3-*0*-rhamnoside isolated from *C. elaeagnoides* had better DPPH radical scavenging activity with IC_{50} of 0.01 µg/mL than the same compound isolated in a mixture with quercetin-3-*0*- rutinoside from *Anacardium occidentale* with IC_{50} value of 0.96 µg/mL (Ajileye et al., 2015). In another study, it was reported that quercetrin (quercetin-3-*0*-rhamnoside) isolated from *Combretum apiculatum* subsp. *apiculatum* had good antioxidant activity with an EC_{50} value of 11.8 µM (0.0053 mg/mL) (Aderogba et al., 2012). These findings support the results of the antioxidant capacity of the similar compound obtained in this study. A preparation such as a plant extract or potentized fraction with multiple functions including antioxidant (to prevent chemical spoilage of food) and antimicrobial (active against both pathogenic and food spoilage organisms) would be of great interest to the food as well as the health industry, as this would enable a reduction in the total amount of additives used in foods (Gutiérrez-Larraínzar et al., 2012).

Table 5. Antioxidant activity of the fractions and isolated compound from *C. elaeagnoides* expressed as mean IC_{50} (µg/mL).

Samples	DPPH	ABTS
CeB	0.16^{a}±0.02	0.01^{a}±0.07
CeH	0.38^{ae1}±0.21	6.28^{b}±0.15
CeD	> 100.00^{b}	20.00^{c}±1.79
CeE	7.00^{c}±0.51	1.44^{d}±0.86
CeH_20	0.06^{a}±0.74	2.81^{e}±0.20
Ceo	0.01^{ae2f1}±0.31	0.90^{f}±1.19
Trolox	2.64^{d}±0.27	0. 74^{f}±0.47
Vitamin C	0.39^{af2}±0.23	0.36^{a}±0.35

CeB (*Combretum elaeagnoides* butanol fraction), CeH (*C. elaeagnoides* hexane fraction), CeD (*C. elaeagnoides* dichloromethane fraction), CeE (*C. elaeagnoides* ethyl acetate fraction), CeH_20 (*C. elaeagnoides* water fraction), Ceo (quercetin-3-*0*-rhamnoside), positive controls (trolox, vitamin C). Across the columns, values with same alphabetical letters are statistically non-significant (P≥0.05) while values with similar alphabetical letters and a different number and values with different alphabetical letters are significantly different (P≤0.05).

3.7 Cytotoxicity and selectivity index values of the fractions and isolated compound from *C. elaeagnoides* leaf extract

Vero cells were specifically selected for cytotoxicity studies as they are a popular cell line to use in routine toxicological studies, owing to their widespread availability, ease of culture and consistency of response to administration of potential toxicants (Fernández Freire et al., 2009). Cell growth or survival, a traditional way to measure cytotoxicity, can be measured in different ways including a change in population size, a change in cell mass, or metabolic activity (McGaw et al., 2014). Most researchers refer to cell viability assays as cytotoxicity assays for practical purposes (McGaw et al., 2014). The results of the cytotoxicity on Vero African monkey kidney cells investigated using the MTT assay, and the selectivity index values, are presented in Table 6. The LC_{50} values of the fractions ranged between 22.20 and 50.26 µg/mL while the isolated compound had an LC_{50} value of 28.17 µg/mL. The water fraction had the highest LC_{50} value of 50.26 µg/mL (lowest toxicity), while the dichloromethane fraction was relatively toxic with an LC_{50} value of 22.20 µg/mL. All the fractions including the isolated compound were less cytotoxic than the

positive control (doxorubicin) which had an LC_{50} value of 15.95 µg/mL against the Vero cells. The statistical analysis showed that there were statistical differences ($P \leq 0.05$) in the cytotoxicity of the fractions and isolated compound when compared to the control (doxorubicin) (Table 6). Zirihi et al. (2005) stated that plant extracts with $LC_{50} \geq 20$ µg/mL are considered relatively safe to cells. A previous study showed that flavonoids generally have low toxicity to animal cells (Havsteen, 2002), and lack of cytotoxicity by the flavonoid 2″-*0*-rhamnosylvitexin was reported against Vero kidney and bovine dermis cells (Aderogba et al., 2007).

Selectivity index (SI) was used as another safety parameter, defined as the ratio of toxicity (LC_{50}) to antibacterial activity (MIC). The dichloromethane fraction had the highest selectivity index value of 0.79 amongst the fractions against *E. coli* **1**. The isolated compound had weak selectivity index values against virtually all the tested foodborne organisms with SI values between 0.0113 and 0.0225. Generally, it is asserted that a high SI value indicates a safer extract. Again, extracts with SI values > 1 are regarded as less toxic to the host cells than to the bacteria. This implies that in this study, the samples were more toxic to the host cells than to the bacteria (Makhafola et al., 2012; Erhabor et al., 2020).

Table 6. Cytotoxicity (LC_{50}) and selectivity index values (LC_{50}/MIC) of the fractions and isolated compound from *C. elaeagnoides* leaf extract.

Samples	LC_{50} (mg/mL)	Selectivity index								
		Ec**1**	Ec **2**	Sa	St	Se	Cj	Sm	Kp	Entero
CeB	0.0484^{a}±1.451	0.1862	0.0387	0.1561	0.0768	0.0465	0.0663	0.0768	0.1308	0.0387
CeH	0.0428^{b}±0.002	0.4756	0.0679	0.0679	0.0171	0..0679	0.1019	0.0679	0.0679	0.0679
CeD	0.0222^{c}±0.140	0.7333	0.0089	0.2220	0.0352	0.0352	0.1057	0.0178	0.0352	0.0089
CeE	0.0249^{d}±0.105	0.4155	0.0396	0.2493	0.0396	0.0396	0.0594	0.0594	0.0804	0.0594
CeH_20	0.0503^{e}±0.005	0.0967	0.0201	0.0598	0.0967	0.0598	0.0201	0.0301	0.0798	0.0402
Ceo	0.0282^{f}±0.669	0.0113	0.0113	0.0113	0.0225	0.0113	0.0113	0.0113	0.0113	0.0113
Doxo	0.0160^{g}±0.127	NA	NA	NA	NA	NA	NA	NA	NA	NA

CeB (*Combretum elaeagnoides* butanol fraction), CeH (*C. elaeagnoides* hexane fraction), CeD (*C. elaeagnoides* dichloromethane fraction), CeE (*C. elaeagnoides* ethyl acetate fraction), CeH_20 (*C. elaeagnoides* water fraction), Ceo (quercetin-3-*0*-rhamnoside), Doxo (Doxorubicin), NA (Not applicable).Across the column, LC_{50} values with same alphabetical letters are statistically non-significant ($P \geq 0.05$) while values with different alphabetical letters are significantly different ($P \leq 0.05$).

4. Conclusions

This is the first-time quercetin-3-*0*-rhamnoside has been isolated and characterized from *Combretum elaeagnoides* leaf extract. The antimicrobial, antibiofilm and antioxidant activity as well as cytotoxicity of the fractions and the isolated compound from *C. elaeagnoides* was also investigated for the first time. The study established that the solvent fractions and isolated compound have reasonably strong antimicrobial, antibiofilm and antioxidant properties, though with varying activities. The solvent fractions and isolated compound were less toxic to mammalian cells than the positive control.

The strong antibiofilm activity of the fractions and particularly the compound, together with their antioxidant activity, supports the traditional use of *C. elaeagnoides* against diarrhoea and other infections. The antibiofilm activity of the fractions and isolated compound could be a possible mechanism of action through which the samples combat foodborne pathogens. The ethyl acetate and dichloromethane fractions had the best MIC and MBC activities while the butanol fraction had the strongest antibiofilm activity. The water fraction had the best DPPH radical radical scavenging potential though not statistically different from the butanol and hexane fraction. The butanol fraction had the best activity against the ABTS radical. Overall, the ethylacetate, dichloromethane and butanol fractions are the most promising fractions. Further mechanistic studies including more intensive investigation of the antibiofilm effects of the most promising fractions and quercetin-3-*0*-rhamnoside, as well as *in vivo* toxicity and efficacy screening, is recommended.

Conflict of interest: Authors declare no conflict of interest.

Authors' contribution: The study is part of the MSc study of RCE supervised by LJM. RCE carried out the work and wrote the first draft. MAA supervised the isolation of compound, JOE and LJM supervised the antibacterial, antibiofilm and antioxidant work while SMN and LJM supervised the cytotoxicity study. LJM, RCE and JOE conceptualized the research.

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