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The Antibacterial, Antioxidant and Phytochemical Composition of *Combretum tanaense* (J. Clark) Root Extracts

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MOJ and AMG designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors MOJ, AMG, KNJ and NAL managed the analyses of the study. Author AWB managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate the antibacterial, antioxidant and phytochemical composition of *Combretum tanaense* extracts.
Study Design: Laboratory-experimental design was used in this study.
Place and Duration of Study: Fresh roots of *Combretum tanaense* were obtained from Mount

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Kenya University botanical garden in Thika (Kiambu County-Kenya). The study was carried out between November 2017 and February 2018 at Mount Kenya University Biochemistry and Pharmacognosy laboratories.

Methodology: Duplicate voucher specimens were prepared and deposited at the East Africa herbarium housed at the National Museums of Kenya and Mount Kenya University herbarium. Extraction of total extracts of *C. tanaense* roots was conducted according to standard procedures. Agar well diffusion and 2-2-diphenyl picryl hydrazyl (DPPH) assay methods were used to evaluate antibacterial and free radical scavenging activities of the extracts. All assays were performed in triplicate. Antibacterial data was presented as a mean zone of inhibition ± SEM while free radical scavenging activities were expressed regarding IC_{50}. Phytochemical screening was carried out using standard procedures to ascertain the presence or absence of various phytochemical groups in the test plant.

Results: The current study indicated that *Combretum tanaense* root extracts had antibacterial activities against the selected gram-positive and gram-negative bacterial strains. The highest activity was recorded against gram-negative bacteria (*Haemophilus influenza)* by exhibiting inhibition zones of 13.32±0.15 mm and 12.82±0.36 mm for methanol and water extracts respectively. Antioxidant activities for both methanol and water extracts were ten times higher compared to that of standard (L-ascorbic acid). The extracts were found to have saponins, phenols including tannins and glycosides.

Conclusion: Extracts of *Combretum tanaense* have compounds that exhibit antibacterial and antioxidant activities. From the results obtained, the ability of the extracts to inhibit bacterial growth and scavenge for free radicals was due to the presence of phenolic compounds and will be attributed to the healing properties of this plant. This study recommends further studies including toxicity and isolation of active compounds for the development of products with pharmaceutical value.

Keywords: Combretaceae; chemotaxonomy; DPPH assay; IC_{50} values; inhibition zones; Kenya.

1. INTRODUCTION

The use of herbal medicine has always been part of human culture for ages because plants harbour important therapeutic properties that cure human and animal diseases. The healing property of medicinal plants is usually attributed to the presence of secondary metabolites that are produced by plants as protective measures against environmental and pathogenic stresses [1].

The secondary metabolites are used as the basis for production of valuable synthetic compounds such as pharmaceuticals, cosmetics and nutraceuticals. It has been reported that a substantial percentage of conventional drug prescriptions contain one or more of bioactive ingredient(s) of plant origin [2]. Secondary metabolites are largely viewed as potential sources of new drugs because of their biological significance and potential health effects, such as antioxidant, antimicrobial, anticancer, anti-ageing, anti-atherosclerotic and anti-inflammatory activities [3].

In the current study, extracts of *Combretum tanaense* were evaluated following the chemotaxonomic approach. The plant belongs to the genus *Combretum*, under Combretaceae family. This genus is the largest in the family and comprises about 250 species with at least 24 of the species being well known in African traditional medicine. Preparations from plants of *Combretum* genus are used for a variety of diseases, including skin infections, wounds dressings and ointments. It is very common to mix different species of *Combretum* or to mix *Combretum* spp. with other medicinal plants for herbal medicine [4].

Kenya traditional medicine practices employ over 22 species of *Combretum,* these are used for treatment of a variety of ailments and disease ranging from scorpion and snake bites, worm remedies, dysentery, fever, headache, microbial infections, abortifacients and aphrodisiacs [5]. It was in this light of many plants from Combretaceae family having a wide range of ethnomedical claims, some of which have been validated, that the current study was conducted to evaluate antibacterial and antioxidant activities as well as the phytochemical groups present in *Combretum tanaense* root extracts.

2. MATERIALS AND METHODS

2.1 Collection and Processing of Plant Materials and Extraction

Fresh roots of *Combretum tananese* plant were harvested from Mount Kenya University botanical garden. The voucher specimens were prepared, deposited at the University herbarium and a duplicate voucher specimen (JMO-2-2015) deposited at the East African Herbarium. The collected roots were cleaned, dried in a, well ventilated room at room temperature away from direct sunlight for one week. Plant materials were washed in tap water to remove unwanted impurities such as grass, soil, and materials of other plants. The roots were also chopped into small pieces to allow for uniform drying and easy grabbling. The dried root materials were ground into moderately coarse powder prior to extraction. Methanol extract was prepared by cold maceration, whereby 100 g of ground powder was soaked in 500 ml of methanol in a 1.5L conical flask for 48 hours with regular shaking. After filtration, the residue was resoaked again using fresh methanol for another 48 hours thrice to ensure exhaustive extraction. The filtrates from methanol extraction were reduced *in-vacuo* using a rotary evaporator at 50°C followed by oven drying at 30°C for complete drying. Water extract was prepared by boiling about 50 g of root powder in water using a boiling beaker at 100°C for five minutes. The aqueous mixture was cooled, filtered and then freeze-dried. All extracts were packed in glass universal sample bottles and kept in a refrigerator at 4°C awaiting bio-assay [6].

2.2 Preparation of Extracts for Antibacterial and Antioxidant Assays

Sample extracts for antibacterial tests were prepared at a concentration of 100 mg/ml using dimethyl sulfoxide (DMSO) as a solvent. Extracts for antioxidant assay were also dissolved in methanol making concentrations of 1000 to 0.01 µg/ml and sonicated for complete dissolution. Ciprofloxacin (0.32 mg/ml) and L-ascorbic acid of the same concentrations as the test extracts were used as a positive control for antibacterial and antioxidant activities.

2.3 Preparation of Culture Media and Inoculates

Nutrient media for growth of the test microorganisms were prepared as per the manufacturer's instructions and sterilized. Each of the subcultured microorganism was suspended in 5 ml of sterilized distilled water and approximately 1.5 ml of the suspension inoculated into 150 ml growth media so as to produce inoculated agar with approximately 1x 10^6 colony forming units per ml. The inoculated nutrient media were then rapidly but carefully poured into Petri dishes using a 25 ml measuring cylinder in such a manner as to deliver 20 ml of inoculated agar with uniform thickness of 3 mm in each petri dish. The layered agar was allowed to cool so as to solidify into a firm gel.

2.4 Agar Well Diffusion Assay

The agar well diffusion method was used to assess antibacterial activities of the crude extracts of *C. tanaense*. Master cultures of *Escherichia coli* (ATCC 10536)*, Staphylococcus aureus* (ATCC 6538)*, Staphylococcus epidermidis, Klebsiella pneumonia, Bacillus subtilis* (ATCC 11778)*, Haemophilus influenzae, Micrococcus luteus* were retrieved from Jomo Kenyatta University microbiology laboratory. Different concentrations of *C. intense* extract were loaded at a volume of 20 µl of sample per 6 mm diameter wells in triplicate and were placed equidistantly on all Petri dishes for all microorganisms. Experiments were done in triplicates and the plates were incubated at 37°C for 18 hrs and results observed. The diameters of the zones of inhibition were then measured using a vernier calipers and tabulated. The images of petri dishes were preserved by photography [7].

2.5 Free Radical Scavenging Activity (DPPH Assay)

Free radical scavenging activities of test extracts was determined using 2-2-diphenyl picryl hydrazyl (DPPH) according to the method described by Ruiz-Teràn et al. with slight modifications [8]. The reaction mixture contained 1.5 ml of *C. tanaense* test extracts or standard (L-ascorbic acid) of different concentrations (1000 µg/ml, 100 µg/ml, 10 µg/ml, 1 µg/ml, 0.1 µg/ml and 0.01 µg/ml) in triplicates and 1.5 ml of 0.3 mM DPPH solution. The reaction mixture was then incubated in the dark for 15 minutes and the absorbance measured at 517 nm against a blank solution containing 1.5 ml of either extract or standard and 1.5 ml of methanol in a Microprocessor UV-VIS Spectrophotometer (Double Beam). The negative control contained 1.5 ml of the 3mM solution of DPPH and 1.5 ml

of methanol. Percentage free radical scavenging activity was determined as follows:

$$\% \text{ RSA} = \{\frac{A0-A1}{A0}\} \times 100\%$$

Where; A_0 is the absorbance of the control and A_1 is the absorbance of either the extract or the standard.

2.6 Phytochemical Screening

Phytochemical tests were conducted for qualitative analysis to determine presence or classes of phytochemical compounds in the powder of *Combretum tanaense* root. Results were evaluated by visual examination of change in colour or precipitation [9,10]. Qualitative chemical tests were conducted as follows:

2.6.1 Test for tannins

Combretum tanaense powder (0.5 g) was boiled in 20 ml of distilled water in a test tube and then filtered. Then 3 drops of 0.5% Ferric chloride ($FeCl_3$) were added to the filtrate. The experiment was observed for bluish-green precipitate.

2.6.2 Test for saponins

2.6.2.1 Foam test

About 0.5 g of *Combretum tanaense* powder was mixed with distilled water (2 ml) in a graduated cylinder and shaken for 3 minutes. observations for production of foam which persisted for ten minutes were recorded.

2.6.2.2 Haemolytic test

Approximately 0.2 g of *C. tanaense* powdered root were extracted with 20 ml of water by heating in a water bath at 70°C for 5 minutes and filtered. Sodium chloride solution 1.8% w/v (2 ml) were placed in a pair of test tubes. In one test tubes, 2 ml of water were added and was labelled control while to the other 2 ml of extract were added and labelled test. To each of the two test tubes, a drop of blood was added, and the test tubes were inverted gently to mix. The test set up as observed for haemolysis in the tube containing the extract and not in control test.

2.6.3 Test for alkaloids

2.6.3.1 Dragendorff's test

Powdered *C. tanaense* root (0.5 g) was extracted with about 8 ml of 1% hydrochloric acid (HCl) with gentle warming in a water bath. The extract was filtered, and then 2 ml of it was treated with Dragendorff's reagent (solution of potassium bismuth iodide). Observations for the appearance of a reddish-brown precipitate were made and recorded as an indication of alkaloids.

2.6.3.2 Meyer's test

The powdered material was prepared as in Dragendorff's test and was treated with Mayer's reagent (potassium mercuric iodide). The experiment was observed for a cream or yellow coloured precipitate which was indicative of alkaloids.

2.6.4 Test for glycosides

2.6.4.1 Test for cardiac glycosides

About 1 g of ground root powder of *C. tanaense* was extracted with 10 ml of 70% alcohol by heating; this was done in a water bath for two minutes using a boiling tube. The obtained extract was allowed to cool and was then filtered. To each filtrate, 10 ml of water was added, and five drops of a strong solution of lead sub acetate and the solution were filtered. To the filtrates, 10% H_2SO_4 was added drop wise until no further precipitation occurred. The resulting solution was filtered and extracted by partitioning with two successful 5 ml portions of chloroform. The two chloroform extracts were combined and washed with 1 ml of distilled water, the washed chloroform extract was filtered and was divided into two equal portions in Petri dishes and evaporated to dryness. Each of the dry extracts was subjected to Kedde and Keller Killian tests.

(i) Kedde test

Two drops of Kedde reagent were added to one portion of the dry extracts in the evaporating dish. The preparation was examined for purple colouration as an indication for the presence of glycosides whose aglycone moiety had unsaturated lactone ring.

(ii) Keller-Kilian test

To the second portion of the dry extracts, 0.4 ml of glacial acetic acid containing trace ferric chloride was added, the preparation was shaken gently to dissolve, and 0.5 ml of concentrated H_2SO_4 was carefully added. Observations for a green-blue colouration in the upper acetic acid layer was indicative of the presence of deoxy sugars.

2.6.4.2 Test for anthraquinone glycosides

(i) Borntrager's test

Powder of *C. tanaense* dry root (0.5 g) was boiled with 5 ml of 10% sulphuric acid (H_2SO_4) in a boiling tube for 5 minutes in a water bath, the extract was filtered while hot, cooled and then shaken with an equal volume of chloroform. The lower layer of chloroform was separated and shaken with dilute ammonia of half its volume. Observation of rose pink to red colour in the ammoniacal layer was considered an indication of anthraquinone glycosides.

(ii) Modified Borntrager's test

About 0.5 g of *C. tanaense* root powder was boiled with 5 ml of 10% H_2S0_4 for minutes in a water bath. Then few drops of 5% ferric chloride added. The mixture was shaken with an equal volume of chloroform. The organic layer was separated and then shaken with the addition of a few drops of dilute ammonia. A rose-pink colour seen in the ammoniacal layer was observed for the indication of anthracene aglycone in a reduced state.

2.6.5 Test for phenols

Approximately 0.5 grams of the material was boiled with 5 ml of 70% alcohol and filtered. To 2 ml of the filtrate, two drops of freshly prepared 5% ferric chloride solution was added; green, blue or violet colourations indicate the presence of phenolic hydroxyl group.

2.7 Data Analysis

Antibacterial activities were analyzed by calculation of the average of the triplicate zones of inhibition of the extracts. The average zone of inhibition was expressed as a mean ± standard error of the mean (SEM) and were compared with the zones of inhibition of the standard antibiotic (Ciprofloxacin). On the other hand, antioxidant activities were analyzed by estimating the concentration of extract that reduced free radicals by fifty percent (IC_{50}). GraphPad Prism version 7.04 statistical software was used to determine to mean ± SEM of the zones of inhibition and IC_{50} values of percentage radical scavenging activities. Student t-test (two-tailed) statistics were performed to determine statistical significance at $p < 0.05$ of antibacterial and antioxidant activities.

3. RESULTS AND DISCUSSION

3.1 Antibacterial Activity of *Combretum tanaense* Root Extract

Water and methanol extracts of *C. tanaense* demonstrated varied ranges of antibacterial activities against the tested microorganisms' concentration of 100 $mgml^{-1}$. According to Alves et al. [11] the microorganisms that show a zone of inhibition of < 9 mm was considered resistant, they included *Staphylococcus epidermidis*, *Bacillus subtilis*, *Micrococcus luteus* for water extract while *Escherichia coli* and *Staphylococcus epidermidis* were equally resistant to methanol extract. The zones of inhibition that measured between 9 mm to 13 mm indicated that the bacterial strains were susceptible towards the extracts as indicated in Table 1. *Staphylococcus aureus, Escherichia coli, Klebsiella pneumonia* and *Haemophilus influenzae* were susceptible to both water and methanol extracts with recorded zones of inhibition between 9 mm to 13 mm. This was indicative of intermediate antibacterial activities towards both gram-negative and gram-positive bacteria. The mean zones of inhibition for the aqueous extracts were not significantly different from those of the methanolic extracts (p = 0.2969). Interestingly, both extracts gave mean zones of inhibition that were significantly different from those of the standard drug (Ciprofloxacin). The statistical difference between methanol extracts had a p-value of 0.0058 while that of water extracts was 0.0001. Antibacterial activities of *C. intense* root extracts are reported for the first time in the current study. However, antibacterial activities of other *Combretum* species has been described and are not limited to *Combretum racemosus* [12], stem bark of *Combretum album* [13], *Combretum micranthum* [14], *Combretum latifolium*, and *C. quadrangular* [15], *C. edwardsii*, *Combretum krausii*, [16] and *Combretum molle* [17].

3.2 Antioxidant Activities of *Combretum tanaense* Extracts

Combretum tanaense extracts demonstrated the potency of scavenging for the 2-2-diphenyl picryl hydrazyl (DPPH) radical activity increased in a dose-dependent manner as it was indicated by decreasing absorbance at 517 nm as with increasing concentration of the extracts. The purple colour of stable DPPH molecule, which was purple, changed to pale yellow and was

Table 1. Antibacterial activity of *Combretum tanaense* root extracts

Microorganism	Mean zone of inhibition in mm		
	Water extract (100 mg/ml)	Methanol extract (100 mg/ml)	Ciprofloxacin (0.32 µg/ml)
Staphylococcus aureus	10.00 ± 0.00	11.50 ± 0.5	14.75 ± 0.25
Staphylococcus epidermidis	8.75 ± 0.25	8.50 ± 0.5	17.75 ± 0.25
Micrococcus luetus	7.3 ± 0.1	7.25 ± 0.05	14.00 ± 0.00
Bacillus subtilis	7.79 ± 0.03	12.75 ± 0.25	14.25 ± 0.25
Escherichia coli	10.00 ± 0.00	7.87 ± 0.03	17.75 ± 0.25
Klebsiella pneumoniae	10.00 ± 0.00	12.25 ± 0.25	16.00 ± 0.00
Haemophilus influenzae	12.82 ± 0.36	13.32 ± 0.15	17.50 ± 0.50

Values are expressed as mean zones of inhibition (mm) ± SEM of triplicate experiments

observed with decreasing intensity of absorbance as shown in figure 1 below. Concentrations of samples that scavenged 50% of the DPPH free radicals (IC_{50}) values was 0.001 µg/ml for both methanol and root extracts of *Combretum tanaense*. This indicated that the extracts were 10 times potent as compared to the standard antioxidant (L-ascorbic acid) whose IC_{50} was estimated at 0.01 µg/ml (Table 2). There were no statistical differences in antioxidant activities between the water and methanol extracts (p = 0.9991) or between the water extract and the standard reference (L-Ascorbic acid) (p = 0.1699) or methanol extract and L-Ascorbic acid (p = 0.0786). In all the experiments, the values for percentage radical scavenging activity were not significantly different at $p<0.05$ as shown in table 3 below. Other workers have found that species from the Combretaceae family also have antioxidant activities including *Combretum roxburghii* [18] and *Combretum rupicola* [19].

Table 2. Concentration of *C. Tanaense* extracts that inhibits scavenge 50 % of free radicals (IC_{50})

Extract	Mean IC_{50} values
C. tanaense methanolic extract	0.001 ± 0.07 µg/ml
C. tanaense aqueous extract	0.001 ± 0.01 µg/ml
L-Ascorbic acid	0.01 ± 0.02 µg/ml

Radical scavenging activities are expressed as Mean ± SEM of IC_{50} (µg/ml) of triplicate experiments

3.3 Phytochemical Screening of *Combretum tanaense*

Results from the current study indicate the presence of different kinds of glycosides and phenolic class of compounds. It was found that alkaloids were absent (Table 3). Out of these phytochemical groups, antibacterial activities reported against both gram positive and gram negative in this study are due to the chemical components that were found in *Combretum tanaense* root extracts. In this case, the antibacterial and antioxidant activities attributed to the presence of phenol containing compounds [20].

Table 3. Phytochemical composition of *Combretum tanaense* roots

Phytochemical	Result
Alkaloids	
Mayer's test	-
Drangendorff test	-
Glycosides	
Borntragers test	+
Modified Borntrager's test	+
Keller-Killian test	+
Kedde test	+
Saponins	
Foaming test	+
Haemolytic test	+
Tannins	+
Phenols	+

Key: + (present); - (Absent)

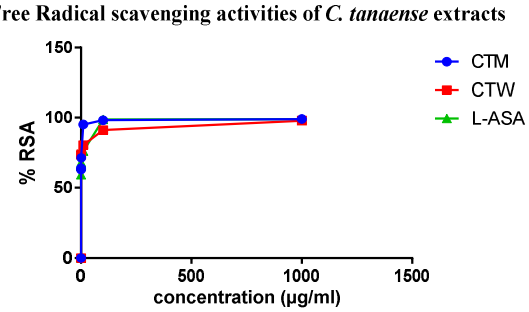

Fig. 1. A graph showing anti-free radical properties of *C. tanaense* root extracts

Key: CTM- Combretum tanaense methanolic root extract; CTW- Combretun tanaense water root extract; L-ASA-L-ascorbic acid

4. CONCLUSION

The current study reports antibacterial and antioxidant activities as well as chemical composition of Kenyan growing *Combretum tanaense* for the first time. The fact that the plant belongs to the family Combretaceae, which is known for a long time to manage number ailments including microbial infections, cancer, snake and scorpion bite, mental problems, heart conditions and worms. It was found that *Combretum tanaense* root extracts have antibacterial and antioxidant activities. However, to the best of our knowledge, the plant does not have any ethnomedical claim either in Kenya or any other part of the world. The study was based on chemotaxonomic knowledge whereby the plant was selected because plant species of Combretaceae family produce compounds or classes of compounds associated with antibacterial and antioxidant activities. Other compounds that were detected in this study were glycosides and tannins; they equally provoke a study on activities that are associated with such group of compounds like anticancer, expectorant, sedative and digestive properties for the former group of phytochemicals and astringents against diarrhoea, anti-inflammatory and anticancer [20]. It is also important to assess the safety of extracts that demonstrated antibacterial and antioxidant activities before any recommendation for use as such. Further studies to isolate the active compounds responsible for the said activities are necessary.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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