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FREE RADICAL SCAVENGING AND ANTIOXIDANT CAPACITY OF LEAF OF *Combretum platypterum* (Welw) Hutch and Dalziel

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Abstract: An antioxidant is a molecule capable of preventing or inhibiting the oxidation of other molecules. It ends these reactions by removing free radical intermediates and inhibiting other oxidation reactions. *Combretum platypterum* is a plant that belongs to the family Combretaceae. Despite being used in ethnomedicine, there is no report of the in vitro antioxidant and free radical scavenging activities of its leaves. This study is aimed at testing the in vitro antioxidant and free radical scavenging activities of *Combretum platypterum* leaves. In vitro antioxidants such as ascorbic acid, carotenoids, and lycopene, and free radical scavenging activities such as 2,2-diphenyl-1-picrylhydrazyl, superoxide radicals, hydroxyl radicals, hydrogen peroxide, nitric oxide, and 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) were analyzed using well-established methods. DNA protective assay was analyzed using DNA unwinding wheat germ plasmtropoisomerase topoi with supercoiled pBR322 plasmid. The vitamin C content in the aqueous extract was significantly higher compared to ethanol, methanol, and chloroform leaf extracts ($p<0.05$). The carotenoid content in the methanol extract was higher compared to ethanol, methanol, and chloroform leaf extracts ($p<0.05$). Lycopene content in the methanol extract was higher compared to water, ethanol, and chloroform leaf extracts ($p<0.05$). DPPH, superoxide radicals, hydroxyl radicals, hydrogen peroxide, nitric oxide, and ABTS are just some of the radicals that the leaves of *Combretum platypterum* can scavenge. Methanol and aqueous extracts of *Combretum platypterum* protected the DNA from damage compared to the effect of dimethyl sulfoxide on DNA. *Combretum platypterum* leaves have antioxidant, scavenging, free radical, and DNA protection potential.

Keywords: *Combretum platypterum*, antioxidant, radicals, vitamin C, carotenoids

1 INTRODUCTION

An antioxidant is a molecule capable of preventing or inhibiting the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. These radicals can start chain reactions that damage cells (Ovuru *et al*., 2023). Antioxidants end these reactions by removing free radical intermediates and inhibit other oxidation reactions. Antioxidants also scavenge the activities of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot}$), hydroxyl radical ($OH^{\cdot}$), hydrogen peroxide (Idu *et al*., 2016; Dysken, 2014; Joseph, 2014). Reactive oxygen species (ROS) cause oxidative damaging by reacting with every molecule in the living cells such as lipid, amino acids, protein and DNA (Suprava *et al.*, 2015). The Reactive oxygen species (ROS) play a vital role in pathogenesis such as Parkinsons disease, hypertension, Atherogenesis, Alzheimers disease, inflammation, asthma, arthritis and cancer. It also plays an important role in ageing (Suprava *et al.*, 2015). Research has shown that plants contain various bioactive part including antioxidant, antihypertensive, antiasthmatic, antiarthritis, anti-inflammation, anticancer and anti-ageing (Erhabor *et al*., 2017; Enerijiofi and Isola, 2019). About 25% of plants active part was identified and prescribed as medicines (Gill *et al*., 2011). The most practical way to fight pathogenesis as a result of reactive oxygen species (ROS) is to increase antioxidant activity in our body and that could be achieved by consumption of vegetables, fruits or edible plants rich in antioxidant

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(Adefegha and Oboh, 2011).

Combretum platypterum belongs to the family of Combretaceae. It occurs from East Guinea to Democratic Republic Congo and Southern Sudan and South to Northern Angola (Idu *et al*., 2016, Dalziel, 1973). It is used to treat lower backache, swellings and mumps, diarrhoea, Blindnsee, cough, headache and fever and as a tonic (Bongers *et al.*, 2005; Neuwinger, 2000). Idu *et al*., 2016 reported *in-vivo* antioxidant activity of aqueous leaf *Combretum platypterum*.

This study are aimed at testing *in-vitro* antioxidant and free radical scavenging activities of leaf *Combretum platypterum*.

Materials and Methods

Plant Material Collection

Fresh leaves of *Combretum platypterum* were harvested from Idumiru, Igbanke West, Orhionmwon Local Government Area, Edo State, Nigeria. The plant was identified and authenticated by Dr. H. Akinnibosun in the Department of Plant and Biotechnology, Faculty of Life Sciences, University of Benin City, Edo State, Nigeria. The plant was deposited in the University of Benin Herbarium with Voucher number UBHc063.

Preparation of Plant Material

Leaves were washed and air-dried in the Department of Plant and Biotechnology, Faculty of Life Sciences, University of Benin, Benin City, for two weeks. The leaves were ground into powder using an impact mill. Pulverized leaves (200g) were macerated with distilled water (5L). After 24 hours, the extracts were filtered, and the filtrate was concentrated over a water bath. The concentrated extracts were stored in universal bottles, labeled, and refrigerated at 4°C before use (Owolabi et al., 2008).

Extraction Using Ethanol, Methanol, and Chloroform

Powdered leaf (150g) was macerated using ethanol, methanol, and chloroform for 72 hours and filtered. The filtrates were oven-dried at 37°C. Each extract of a different solvent was stored in a glass container in a refrigerator until use.

Determination of In-Vitro Antioxidant Activities

The in-vitro antioxidant activities of aqueous, methanol, ethanol, and chloroform leaf extracts of *Combretum platypterum* were determined as follows:

Determination of Ascorbic Acid

The plant extract (0.1g) was mixed with 1mL of 4% TCA. A centrifugation process was performed at a speed of 2000 revolutions per minute for a duration of 10 minutes. The liquid obtained was mixed with a small amount of activated charcoal, vigorously shaken, and left for 5 minutes. Centrifugation eliminated the charcoal particles. Supernatant (0.5mL) was added to 2mL of 4% trichloroacetic acid (TCA), followed by the addition of 0.5mL of 2% dinitrophenylhydrazine (DNPH) in 9-normal sulfuric acid (H_2SO_4), and then 2 drops of 10% thiourea solution. The components were combined and kept at a temperature of 37°C for 3 hours, which led to the formation of formosazone crystals. The crystals were dissolved in 2.5mL of 85% sulfuric acid at a low temperature. After adding sulfuric acid, DNPH reagent and thiourea were added to the blank. The tubes were cooled in ice, and the absorbance was measured at 540nm using a spectrophotometer (Model: T80+UV/Vis Spectrometer, PG Instruments Ltd.). The outcome was indicated as milligrams per gram of the sample.

Determination of Total Carotenoids and Lycopene

The determination was carried out in the dark to avoid the photolysis of carotenoids. Plant extract (0.1g) was homogenized and saponified with 0.5mL of 12% alcoholic potassium hydroxide in a water bath at 60°C for 30 minutes. The saponified extract was transferred to a separating funnel containing 3mL of petroleum ether and mixed well. The lower aqueous layer was transferred to another separating funnel, and the upper petroleum ether layer containing the carotenoids was collected. The extraction was repeated until the aqueous layer became colorless. Little quantities of anhydrous sodium sulfate were added to the petroleum ether extract to remove excess moisture. The final volume of the petroleum ether extract was noted. Absorbance of the yellow color was read in a spectrophotometer (Model: T80+UV/Vis Spectrometer, PG Instruments Ltd.) at 450nm and 503nm using petroleum ether as the

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blank. The amount of total carotenoids and lycopene was calculated using the formula (Raghunathan and Matheswaran, 2016; Nithya et al., 2015).

$$\text{Amount of total carotenoids} = \frac{A_{450} \times \text{sample} \times 100 \times 4}{\text{Weight of the sample}}$$

$$\text{Amount of lycopene} = \frac{3.12 \times A_{503} \times \text{ sample} \times 100}{\text{Weight of the sample}}$$

The total carotenoids and lycopene were expressed as mg/g of the sample.

Evaluation of Radical Scavenging Activity

The scavenging effects of aqueous, methanol, ethanol, and chloroform leaf extracts of *Combretum platypterum* were tested against 2,2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide, superoxide, nitric oxide, and hydroxyl radicals.

2,2-Diphenyl-1-picrylhydrazyl Hydrate (DPPH) Scavenging Effects

The scavenging abilities of the natural antioxidants in the leaves towards the stable free radical DPPH were measured following the method of Mensor et al. (2001). Aqueous, methanol, ethanol, and chloroform leaf extracts at various concentrations were added to 1mL of the methanol solution of DPPH. The mixture was allowed to react for 30 minutes at 25°C. Methanol served as the blank, DPPH in methanol as a control, and ascorbic acid as the standard. After 30 minutes of incubation, the discoloration of the purple color was measured at 518nm in a spectrophotometer (Model: T80+UV/Vis Spectrometer, PG Instruments Ltd.). The free radical scavenging activity was calculated as follows:

$$\text{Inhibition (\%)} = \left(\frac{\text{Control} - \text{Test}}{\text{Control}}\right) \times 100$$

Measurement of Superoxide Scavenging Activity

The various concentrations of aqueous, ethanol, methanol, and chloroform leaf extracts were added to 0.2mL of EDTA, 0.1mL of NBT, 0.05mL of riboflavin, and 2.64mL of phosphate buffer. The control tubes were set up, and distilled water was added instead of the plant extracts. Optical density was measured at 560nm in a spectrophotometer (Model: T80+UV/Vis Spectrometer, PG Instruments Ltd.) (Shinde et al., 2006). Superoxide anion scavenging activity was calculated as follows:

$$\text{Inhibition (\%)} = \left(\frac{\text{Control} - \text{Test}}{\text{Control}}\right) \times 100$$

Hydrogen Peroxide Scavenging Effects

The leaf extracts (10mg/10L) of aqueous, methanol, ethanol, and chloroform were added to 0.6mL of combined H_2O_2 (40mM) in phosphate buffer, and the total volume was made up to 3mL. The absorbance of the reaction mixture was recorded at 230nm in a spectrophotometer (Model: T80+UV/Vis Spectrometer, PG Instruments Ltd.). A blank solution containing phosphate buffer without H_2O_2 was prepared (Shinde et al., 2006; Ruch et al., 1989). The extent of H_2O_2 scavenging in the plant extracts was calculated as:

$$\%\text{scavenging of hydrogen peroxide} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

where A_0 is the absorbance of control and A_1 is the absorbance in the presence of plant extract.

Measurement of Hydroxyl Radical Scavenging Activity

Hydroxyl radical scavenging activity was assessed according to Uwaya et al. (2021). The reaction mixture contained 0.1mL of deoxyribose, 0.1mL of $FeCl_3$, 0.1mL of EDTA, 0.1mL of H_2O_2, 0.1mL of ascorbate, and 0.1mL of KH_2PO_4-KOH buffer. The mixture was incubated at 37°C for 1 hour. At the end of the incubation period, 1.0mL of TBA was added and heated at 95°C for 20 minutes to develop the color. After cooling, the TBARS formation was measured spectrophotometrically at 532nm against the blank. The hydroxyl radical scavenging activity was determined as:

$$\text{Inhibition (\%)} = \left(\frac{\text{Control} - \text{Test}}{\text{Control}}\right) \times 100$$

Measurement of Nitric Oxide Scavenging Activity

The reaction was initiated by adding 2.0mL of sodium nitroprusside, 0.5mL of PBS, and 0.5mL of leaf extracts of aqueous, methanolic, ethanolic, and

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chloroform, which were incubated at 25°C for 30 minutes. Griess reagent (0.5mL) was added and incubated for another 30 minutes. The control tubes were prepared without the extracts. The absorbance was read at 546nm against the reagent blank in a spectrophotometer (Uwaya et al., 2021).

2,2-Azinobis (3-Ethylbenzothiazoline-6-Sulphonic Acid) Radical Scavenging Activity

To make ABTS+ stock solution, 7mM ABTS aqueous solution was mixed with 2.45mM potassium persulfate aqueous solution in an equal amount. The mixture was then left to sit at room temperature in the dark for 12 to 16 hours before it was used. The working solution of ABTS+ was obtained by diluting the stock solution in methanol to give an absorbance of 0.70 at 734nm. Then, 2.0mL of ABTS+ solution was mixed with 1mL of various concentrations of extract of different solvents of *Combretum platypterum* leaves (10–100μg/mL). The mixture was then incubated at room temperature for 10 minutes in the dark. The control was prepared by mixing 2.0mL of ABTS+ solution with 1mL of double-distilled water. The absorbance was measured against a blank at 734nm using a spectrophotometer. Ascorbic acid was used as the standard. The percentage of scavenging activity of each extract on ABTS+ was calculated as inhibition (I%) using the following equation:

$$I\% = \left(\frac{A_o - A_s}{A_o}\right) \times 100$$

(Russo et al., 2000; Jayashree et al., 2015).

Reactions of Wheat Germplasm Topoisomerase I with Supercoiled pBR322 Plasmid

A 50μL solution containing 35mM Tris-HCl (pH = 8), 72mM KCl, 5mM $MgCl_2$, 5mM DTT, 5mM spermidine, 100μg/kg of aqueous and methanol extract leaves *Combretum platypterum*, 200 to 1000ng pBR322 plasmid, and 2U/μL Wheat Germplasm Topoisomerase I was incubated with or without DMSO, ethidium bromide at 37°C for 30 minutes. One unit is defined as the amount of enzyme that catalyzes 100% of 0.5μg of pBR322 (relaxed and supercoiled) DNA in 30 minutes at 37°C in a total reaction volume of 50μL. The obtained products were further analyzed using agarose electrophoresis (1.0%) without ethidium bromide. The gel was photographed, and the DNA bands were measured (Bei et al., 2015; Forte et al., 1989).

Statistical Analysis

The data were expressed as the mean ± SEM. The data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. Statistical analysis was performed using GraphPad Prism V.6.01, where $p < 0.05$ was considered significant.

Results

Vitamin C, Carotenoid, and Lycopene Content

The amounts of vitamin C, carotenoids, and lycopene in water, ethanol, methanol, and chloroform leaf extracts of *Combretum platypterum* are shown in Figures 1, 2, and 3. Vitamin C content in the aqueous leaf extract is significantly higher compared to ethanol, methanol, and chloroform leaf extracts (pi0.01) (Figure 1). The carotenoid and lycopene contents in the methanol extract were significantly higher compared to aqueous, ethanol, and chloroform leaf extracts (pi0.01) (Figures 2 and 3).

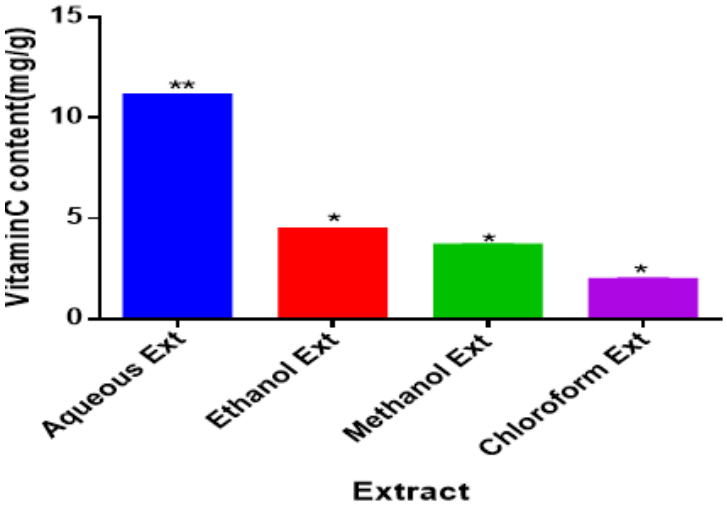

Figure 1: Vitamin C content in aqueous, ethanol, methanol, and chloroform leaves extract of *Combretum platypterum*.

Radical Scavenging Activity of Leaf Extracts

Figures 4, 5, 6, 7, and 8 show the DPPH, superoxide, hydroxyl, hydrogen peroxide, nitric oxide, and ABTS radical scavenging activities of the aqueous, ethanol, methanol, and chloroform leaf extracts of *Combretum platypterum*. Aqueous leaf extract showed the best

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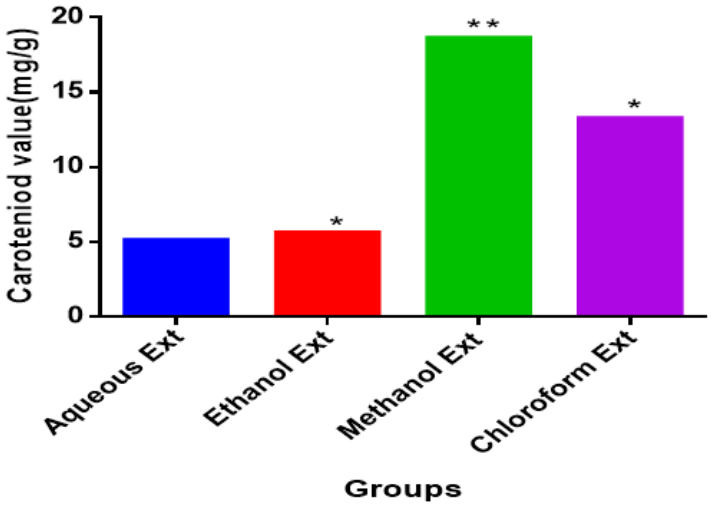

Figure 2: Carotenoid content in aqueous, ethanolic, methanolic, and chloroform leaves extract of *Combretum platypterum*.

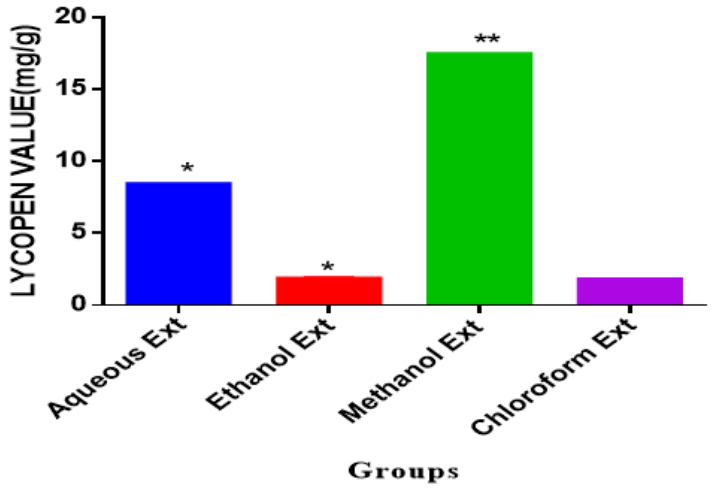

Figure 3: Lycopene content in aqueous, ethanol, methanol, and chloroform leaves extract of *Combretum platypterum*.

DPPH, superoxide, hydroxyl, hydrogen peroxide, and ABTS radical scavenging activities compared to the standard vitamin C (Figures 4, 5, 6, 7, and 9). The ethanol extract gave the best nitric oxide radical scavenging activity (Figure 8).

DNA Unwinding Assay

Discussion

Plants are potential sources of compounds needed to develop new therapeutic agents (Suprava et al., 2013; Adesina et al., 2013). They play an important role in effective antioxidant and free radical scavenging activities (Suprava et al., 2013). The *in vitro* antioxidant study showed that *Combretum platypterum* leaves contain vitamin C, carotenoids, and lycopene. Vitamin C content was highest in the aqueous extract. Carotenoid and lycopene were highest in the methanolic extract. Vitamin C, carotenoid, and lycopene are powerful antioxidants and play a vital role in disease prevention and treatment (Kulawik et al., 2023; Chávez-Mendoza et al., 2015).

Vitamin C is a strong antioxidant, and small amounts can protect molecules in the body, such as proteins, lipids, carbohydrates, and nucleic acids, from damage by free radicals and reactive oxygen species that can be generated during normal metabolism and through exposure to toxins and

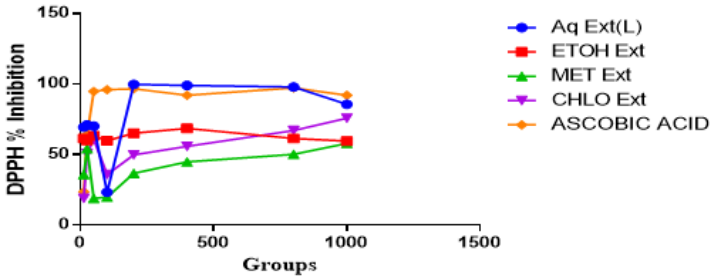

Figure 4: DPPH radical scavenging activities of aqueous, ethanol, methanol and chloroform leaves extract of *Combretum platypterum*.

Figure 4: DPPH radical scavenging activities of aqueous, ethanol, methanol, and chloroform leaves extract of *Combretum platypterum*.

pollutants. Vitamin C may also regenerate other antioxidants, such as vitamin E (Bruno et al., 2006). Vitamin C is beneficial in preventing the onset of chronic diseases such as cancer or heart disease (Peng, 2014). The high content of vitamin C in the aqueous leaf extract of *Combretum platypterum* lends credence to the young leaves being used as soup in Sierra Leone (Bongers et al., 2005; Burkill, 1985).

Carotenoid is a powerful antioxidant. It protects against many degenerative conditions, including heart disease, diabetes, cancer, macular degeneration, cataracts, arthritis, and skin damage, and promotes male fertility (Perusek and Maeda, 2013; Wang et al., 2013). Lycopene is a carotenoid that is linked to maintaining the health of the liver, colon,

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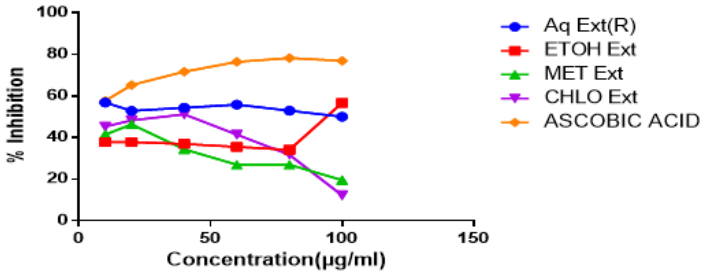

Figure 5: Superoxide radical scavenging activities of aqueous, ethanolic, methanolic, and chloroform leaves extract of *Combretum platypterum*.

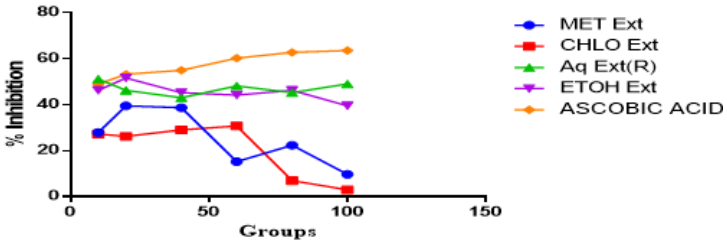

Figure 6: Hydroxyl radical scavenging activities of aqueous, ethanol, methanol, and chloroform leaves extract of *Combretum platypterum*.

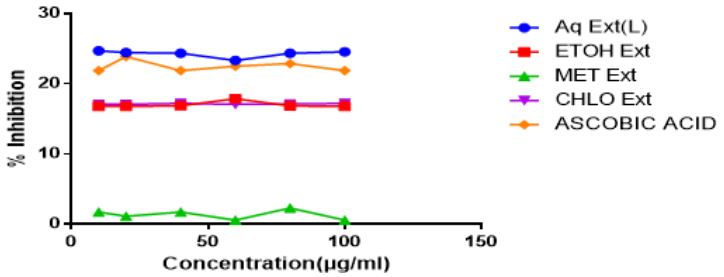

Figure 7: Hydrogen peroxide radical scavenging activities of aqueous, ethanolic, methanolic, and chloroform leaves extract of *Combretum platypterum*.

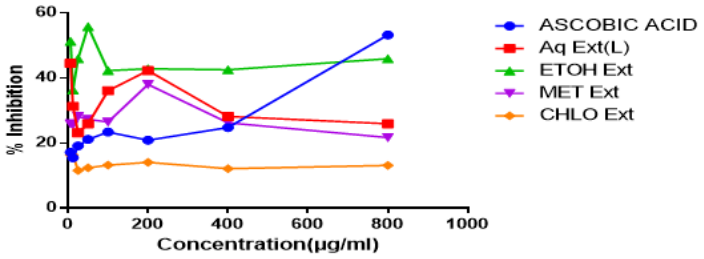

Figure 8: Nitric oxide radical scavenging activities of aqueous, ethanolic, methanolic, and chloroform leaves extract of *Combretum platypterum*.

breast, prostate, and lungs. It prevents osteoporosis, diabetes, and cancer, treats infertility in men, and protects cells by inhibiting apoptosis (Trejo-Solís et al., 2013; Sadek et al., 2016). *Combretum platypterum* leaves are rich in vitamin C, carotenoids, and lycopene, which gives credence to its use in traditional medicine to treat inflammation, pain, cough, fever, eye problems, lumps, conjunctivitis, sexually transmitted diseases, diarrhea, and as a tonic (Liben, 1983; Bongers et al., 2005).

A 2-diphenyl-2-picrylhydrazyl hydrate scavenging assay showed that *Combretum platypterum* leaves can scavenge free radicals generated by oxidative stress. The effect of the leaf extract in different solvents is comparable to that of vitamin C. 2-diphenyl-2-picrylhydrazyl hydrate is a stable and available organic nitrogen radical that is mostly used to determine antioxidant activity (Kamra et al., 2012; Huang et al., 2005). The scavenging ability can be attributed to the presence of vitamin C, carotenoids, and lycopene in *Combretum platypterum* leaves. Aqueous leaf extract showed the best superoxide radical scavenging activity compared to the standard vitamin C. The hydroxyl radical scavenging activity of aqueous, ethanolic, methanolic, and chloroform leaf extracts showed the ability to scavenge hydroxyl radicals. Aqueous leaf extract shows the best hydroxyl radical scavenging activity, comparable to the standard vitamin C. Hydroxyl radicals are the most dangerous among the radicals. They react with most biomolecules, such as proteins, nucleic acids, lipids, and polypeptides, causing tissue damage and cell death. Scavenging hydroxyl radicals is a vital antioxidant mechanism for protecting living cells (Rajagopalan et al., 2014).

In terms of hydrogen peroxide radical scavenging activity, aqueous leaf extract showed the best activity. This may be due to the high content of vitamin C in the aqueous leaf extract. Nitric oxide radicals are produced in various inflammatory conditions, such as juvenile diabetes, multiple sclerosis, arthritis, ulcerative colitis, and carcinomas. Nitric oxide reacts with superoxide radicals to form a highly reactive peroxynitrite anion (ONOO-), which is toxic (Rajagopalan et al., 2014). At lower concentrations, the nitric oxide radical scavenging activity of aqueous, ethanolic, and methanolic solutions was higher than

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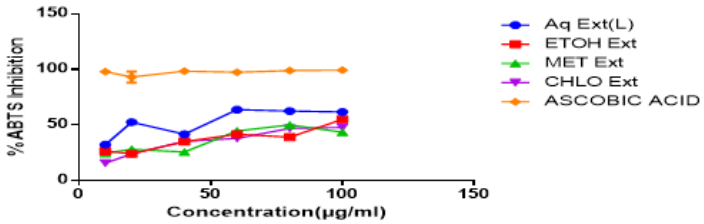

Figure 9: ABTS scavenging activity of aqueous, ethanolic, methanolic, and chloroform leaves extract of *Combretum platypterum*.

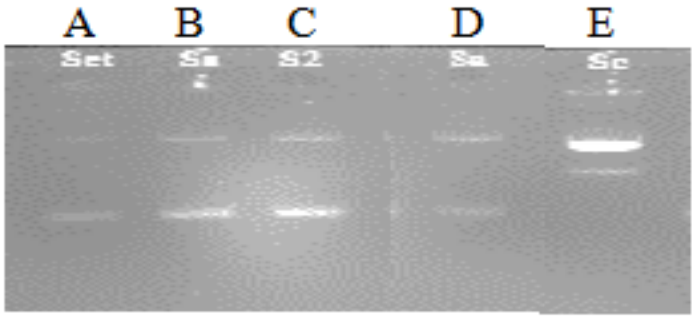

Figure 10: DNA unwinding assay using Wheat Germplasm Topoisomerase 1 exposed to Supercoiled Plasmid DNA (pBR322). Lane A is supercoiled + ethidium bromide, Lane B is Supercoiled + water, Lane C is supercoiled + DMSO, Lane D is Supercoiled + aqueous leaves extract of *Combretum platypterum*, and Lane E is Supercoiled + methanolic leaves extract of *Combretum platypterum*.

that of vitamin C. The leaf extract has the ability to prevent the generation and scavenging of superoxide radicals, protecting individuals from their harmful effects. Aqueous leaf extract shows the best ABTS scavenging ability; this may be due to the high content of vitamin C.

The scavenging ability of *Combretum platypterum* leaves in various solvents showcases the rich antioxidant properties of the plant. Antioxidants help to remove reactive oxygen species, which may lead to oxidative stress. This imbalance leads to the damage of vital biomolecules and cells, potentially impacting organisms (Durackova, 2010). The presence of vitamin C, carotenoid, and lycopene, and the scavenging potentials of DPPH, superoxide radicals, hydroxyl radicals, hydrogen peroxide, nitric oxide radicals, and ABTS in the leaf extract of

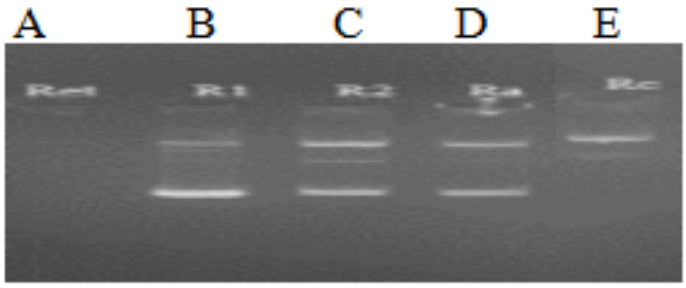

Figure 11: DNA unwinding assay using Wheat Germplasm Topoisomerase 1 exposed to the relaxed form of plasmid DNA (pBR322). Lane A is Relaxed + Wheat Germplasm Topoisomerase 1 + ethidium bromide, Lane B is Relaxed + Wheat Germplasm Topoisomerase 1 + water, Lane C is Relaxed + Wheat Germplasm Topoisomerase 1 + DMSO, Lane D is Relaxed + Wheat Germplasm Topoisomerase 1 + aqueous leaves extract of *Combretum platypterum*, and Lane E is Relaxed + Wheat Germplasm Topoisomerase 1 + methanolic leaves extract of *Combretum platypterum*.

Combretum platypterum show that the plant has the ability to protect cells from being damaged by oxidative stress.

A DNA protection unwinding assay using wheat germplasm topoisomerase I exposed to supercoiled plasmid DNA (pBR 322) and relaxed plasmid DNA showed that the aqueous and methanolic leaf extracts had a protective effect on DNA compared to the effects of dimethyl sulfoxide (DMSO) and water. Dimethyl sulfoxide at low concentrations protects DNA by minimizing the formation of OH-induced sugar and base damage (deLara et al., 1995). Hydroxyl free radicals cause 60–70% of cellular DNA damage (Mira and Pawel, 2012). The scavenging effect of the leaf extract of *Combretum platypterum* on hydroxyl radicals indicates its protective effect on DNA.

Conclusion

Combretum platypterum leaves possess antioxidant and free radical-scavenging activities. Additionally, it demonstrated the ability to shield DNA from free radical damage. The antioxidant and free radical-scavenging activities of *Combretum platypterum* leaves lend credence to their use in treating various ailments in ethnomedicine.

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Conflict of Interest

The authors declare no conflict of interest.

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