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Research Article
Antioxidant Activity of Partially Purified Fractions of *Daniellia oliveri* (Rolfe) Hutch and Dalziellii Stem Bark Ethanolic Extract

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Abstract

Background and Objective: Oxidative stress has been an underline cause of many disease conditions which come into play using different mechanistic steps. Antioxidants and other natural mechanisms have been put in place to mitigate the initiation and/or propagation of these mechanisms and further translation to their effects. This study was designed for *in vitro* assessment of antioxidant claims of the plant and to identify the specific fraction responsible for the acclaimed effects using different methods. **Materials and Methods:** Fractionation was achieved by column chromatography using solvents of different polarities to obtain fourteen fractions, following which Total Antioxidant Capacity (TAC), Total Flavonoid Contents (TFC), Total Phenolic Content (TPC), β-carotene bleaching inhibition assay, anti-lipid peroxidation inhibition assay and metal-chelating inhibition assay were conducted. **Results:** Antioxidant activities of fractions expressed in mg mL^{-1} of Trolox Equivalent (TE). Values ranged from 0.03-0.13 mg mL^{-1}. The total flavonoid concentration varied from 15.71-29.20 mg mL^{-1} expressed as quercetin equivalent. The β-carotene bleaching inhibition assay varied from 21.20-89.60 μg mL^{-1}, while anti-lipid peroxidation inhibition assay and metal-chelating assay results varied from 13.98-41.63 nmol mL^{-1} and 382.53-412.27 g mL^{-1}, respectively. Linear correlation analysis between the parameters revealed a weak positive relationship. **Conclusion:** Based on the results, it is concluded that fractions of ethanolic extract of *D. oliveri* stem bark, particularly fractions 11 and 12, possess potent antioxidant activity irrespective of parameters used, suggesting that this extract contains substances that could act through synergistic action related to, but not limited to direct free radical scavenging, chelation of transition metals and direct inhibition of lipid peroxidation.

Key words: Estimation, antioxidant, fractions, ethanolic, *Daniellia oliveri*, radical scavenging, chelation

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Oxygen is an element obligatory for life, living systems have evolved to survive in the presence of molecular oxygen and for most biological systems[1]. Oxidative properties of oxygen play a vital role in diverse biological phenomena. Oxygen has double-edged properties, being essential for life, it can also aggravate the damage within the cell by oxidative events[2]. Free radicals are well documented for playing a dual role in our body as both deleterious and beneficial species. In low/moderate concentrations free radicals are involved in normal physiological functions but excess production of free radicals or a decrease in antioxidant level leads to oxidative stress[3,4].

Antioxidants are both natural and synthetic compounds that can scavenge free radicals and inhibit oxidation processes[5]. Many synthetic antioxidants such as butylated hydroxyanisole (BHA) and Butylated Hydroxytoluene (BHT) are very effective and used for industrial processes, but they possess some side effects and toxic properties in human health, thus warranting the increasing interest in natural antioxidants, e.g., polyphenols, present in medicinal and dietary plants[6].

Medicinal plant parts are commonly rich in phenolic compounds, such as flavonoids, phenolic acids, stilbenes, tannins, coumarins, lignans and lignins. These compounds have multiple biological effects including antioxidant activity[7]. Antioxidants are widely used in dietary supplements and have been investigated for the prevention of diseases such as cancer, coronary heart disease and even altitude sickness[8-11] since earlier studies suggested that antioxidant supplements have health-promoting potentials[12,13].

Daniellia oliveri leaf and stem bark are used in Northern Nigeria for a variety of gastrointestinal complaints and diabetes mellitus. The resin of this plant is used to heal sores and against microbial infections. An earlier study evaluated the effectiveness of the use of *Daniellia oliveri* stem bark as a remedy for gastrointestinal parasites[14,15]. Also, the leaf of *Daniellia oliveri* has been found effective as an antispasmodic agent, *in vitro* on isolated guinea pig ileum[16].

Therefore, the determination of the phytochemical properties and antioxidant potential of the plant stem bark was considered important as a means of providing more data on the pharmacological properties of the plant. This investigation was a comparative study of different methods of determining of antioxidant activities of fractions of ethanolic extract of *Daniellia oliveri* stem bark.

MATERIALS AND METHODS

Study area: This research project was conducted from November, 2016 to August, 2017 at the Federal University, Wukari, Nigeria.

Sample collection: The healthy stem bark of the plant, *Daniellia oliveri* was collected from Biological Garden within the Federal University, Wukari Campus, Wukari LGA, Taraba State, Nigeria. The plant was authenticated by Jemilat Ibrahim, a Taxonomist with the Ethnobotany and Herbarium Section, National Institute for Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria where a Voucher Number: NIPRD H5483 was assigned.

Sample preparation: The stem barks were examined to be free from diseases. The stem bark was cut into pieces using a kitchen knife and was dried under shade until it became brittle before being pulverized using laboratory mortar and pestle.

Ethanolic extraction: Pulverized sample of *Daniellia oliveri* stem bark (100 g) was soaked in 400 mL of ethanol (1:4 w/v) and was allowed to stand for 48 hrs at room temperature according to the method described by Iwueke and Nwodo[17]. The extracts were filtered out first, using a clean white sieving mesh and then using the Whatman No. 1 filter paper. The concentrated extracts were then transferred to air-tight containers, corked and preserved in the refrigerator at 4°C until required. Aliquots of the crude plant extract residue were weighed and used for phytochemical screening.

Fractionation of ethanolic extract: The ethanol extract was subjected to column chromatography to separate the extract into its component fractions using the method earlier described by Yakubu *et al.*[18]. Silica gel was used in packing the column while solvent combinations of varying polarities were used as the mobile phase.

Packing of the column: The lower part of the glass column was stocked with glass wool with the aid of a glass rod. Silica gel of G_{60-200} mesh size (75 g) was dissolved in 180 mL of absolute ethyl acetate to make the slurry. The chromatographic column (30 mm diameter by 40 cm height) was packed with silica gel and allowed the free flow of the solvent into a conical flask. At the end of the packing process, the tap was locked and the column was allowed 24 hrs to stabilize after which, the clear solvent at the top of the silica gel was allowed to drain down the silica gel meniscus.

Elution: The extract (2 g) was dissolved in 2 mL absolute methanol and the solution was applied to a chromatographic column. Elution of the extract was done with a solvent system of gradually increasing polarity, beginning from chloroform, ethyl acetate, methanol and finally water. The following ratio of solvent combinations was sequentially used in the elution process, ethyl acetate 100:00, ethyl acetate:ethanol 50:50, ethyl acetate:ethanol 50:50, ethanol:methanol 50:50, ethanol:methanol 0:100, methanol:water 50:50, methanol:water 0:100.

The column was eluted with various eluents (400 mL each time) and the fractions were collected in aliquots of 200 mL.

Determination of total antioxidant capacity (TAC): The 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was measured in triplicates based on the procedure of Augustine *et al.*[19]. This is based on the principle that, because of its odd electron, DPPH interact with the compounds (antioxidants in the sample) to reduce the radicals to corresponding hydrazines, changing the DPPH colour from violet to yellow with the intensity being proportional to the radical scavenging activity and is measured at 517 nm. Total antioxidant capacity (TAC) was calculated as mg mL^{-1} trolox equivalent (TE) using the regression equation from the calibration curve constructed from trolox standard concentration (25, 50, 100, 150 and 250 mg mL^{-1}).

Determination of total flavonoids content: Total flavonoids were determined using the aluminium chloride colorimetric method[20]. Quercetin standard was used for derivation of the calibration curve (50, 100, 150, 250 and 500 mg mL^{-1}). Total flavonoids were expressed as mg mL^{-1} quercetin equivalent (QE).

Determination of total phenolic compounds: Total phenolic content (TPC) of the extract was estimated following the phosphomolybdic/phosphotungstic acid complex procedure[21] utilizing the Folin-Ciocalteu (FC) reagent. The method relies on the transfer of electrons in an alkaline medium from phenolic compounds to form a blue chromophore constituted by a phosphotungstic/phosphomolybdenum complex where the maximum absorption depends on the concentration of phenolic compounds. The reduced Folin-Ciocalteu reagent is detectable with a spectrophotometer in the range of 690-710 nm. The reaction temperature (35°C) was used to reduce the time necessary to attain the maximum colour. The measurements were compared to a standard curve of prepared gallic acid solutions (20, 40, 60, 80 and 100 mg mL^{-1}) and expressed as milligrams of gallic acid equivalents.

β-carotene bleaching inhibition assay: The antioxidant activity was determined by measuring the inhibition of conjugated diene hydroperoxides arising from linoleic acid oxidation by reacting it with β-carotene[22]. The stock solution of the β-carotene-linoleic acid mixture was prepared thus, 0.5 mg β-carotene was dissolved in 1 mL of chloroform. Thereafter, 25 µL linoleic acid and 200 mg Tween 40 were added. Chloroform was then evaporated using a vacuum evaporator. Then 100 mL of oxygenated distilled water was added with vigorous shaking, 2.5 mL of this reaction mixture was transferred into test tubes and 0.35 mL of the extracts (2 mg mL^{-1}) were added, the emulsion system was incubated for 2 hrs at 50°C and the absorbance of the mixtures was determined at 490 nm.

Measurement of absorbance was continued until the colour of β-carotene disappeared. The bleaching rate (R) of β-carotene was calculated according to the equation:

$$R = \frac{\ln (a/b)}{t}$$

Where:
ln = Natural log
a = Absorbance at time 0
b = Absorbance at time t (120 min)

Metal chelating activity: The metal chelating activity of the extract fractions with ferrous ions was measured in triplicates following the method of Johnson *et al.*[23]. The reaction mixture containing 0.5 mL of the fraction, 1.6 mL of water, 0.05 mL of $FeCl_2$ (2 mM) and 0.1 mL of ferrozine (5 mM) was incubated at 40°C for 10 min and the absorbance was measured at 562 nm. Measurement was carried out in triplicate for all fractions. The chelating activity of the extract at different concentrations was calculated as:

$$\text{Chelating activity (\%)} = \frac{A_1 - A_2}{A_0} \times 100$$

Where:
A_0 = Absorbance of the control (without extract)
A_1 = Absorbance of the reaction mixture
A_2 = Absorbance without $FeCl_2$

Lipid peroxidation inhibition assay: This assay was used to estimate the degree of lipid peroxidation of the plant extract by following the method of Yakubu *et al.*[24]. Homogenate (10%) was prepared from the freshly excised liver of a healthy goat using cold phosphate buffer saline (pH 7.4). Fraction (0.1 mL) and 2.8 mL of the freshly prepared 10% liver

homogenate were added to 0.1 mL of (50 mM) ferrous sulphate and incubated for 30 min following which 0.1 mL of the reaction mixture was mixed with 1.5 mL of 10% trichloroacetic (TCA) and further incubated for 10 min. It was then filtered and the supernatant was added to a tube containing 1.5 mL of 0.67% thiobarbituric acid (TBA) (in 50% acetic acid) and placed in a boiling water bath for 30 min. The colour developed by the different concentrations was measured at 535 nm. Measurement was carried out in triplicate for all fractions. Anti-lipid peroxidation was assessed by using the following formula:

$$\text{Lipid peroxidation inhibition (\%)} = \frac{(A_i - A_s)}{(A_i - A_c)} \times 100$$

Where:

A_i = Absorbance of Fe^{2+} induced peroxidation
A_s = Absorbance of test sample
A_c = Absorbance of control

RESULTS

Figure 1 shows the total antioxidant capacity of different fractions of the extract. Fractions 11 and 12 possessed the highest antioxidant activity followed by fractions 13, 14, 6, 5 and 4. Fractions 7, 8 and 9 have the same TAC, while fractions 2, 1 and 3 have the least.

In Fig. 2, fractions 11 and 12 have the highest TFC concentrations followed by fraction 4 and continued in the following order of fractions, 1>9>5>6>8>3>13>14>7>10 and finally 2.

Total phenolic content (TPC) concentrations ranged from 1453.85-1460.77 mg mL^{-1} GAE with fractions 11 having the highest concentration and 1, 3 and 6 having the lowest concentration as depicted in Fig. 3.

The results for the β-carotene bleaching assay showed a different pattern from that of TAC, TFC and TPC. In the β-carotene bleaching assay, fraction 8 has the highest activity and then continues in the following order, 9>5>1>2>4, with fraction 10 having the least in Fig. 4.

Thiobarbituric Acid Reactive Substances (TBARS) results show that fractions, 11, 12, 6 and 5 distinctively showed the highest anti-lipid peroxidation level compared to other fractions. Hence, fractions 1, 2, 9, 13 and 14 have the least TBARS concentrations in Fig. 5.

The metal chelating assay revealed that almost all the fractions have the same activity with insignificant ($p>0.05$) changes in concentration between the fractions, except fraction 14 which showed the least level in Fig. 6.

Correlation analysis between the parameters revealed the following: TAC/TFC ($R^2 = 0.0147$), TAC/TPC ($R^2 = 0.1314$) and TAC/β-carotene ($R^2 = 0.0966$) in Fig. 7-9 were all weak positive relationship. While that of TBARS/TFC ($R^2 = 0.0466$) and metal chelating/TFC ($R^2 = 0.0007$) in Fig. 10 and 11 showed extremely weak relationship.

DISCUSSION

The total antioxidant capacity of the fractions obtained from the ethanolic extract of *Daniellia oliveri* stem bark ranged from 0.03-0.13 mg mL^{-1} (Fig. 1), indicating that plant extract has an appreciable amount of bioactive compounds.

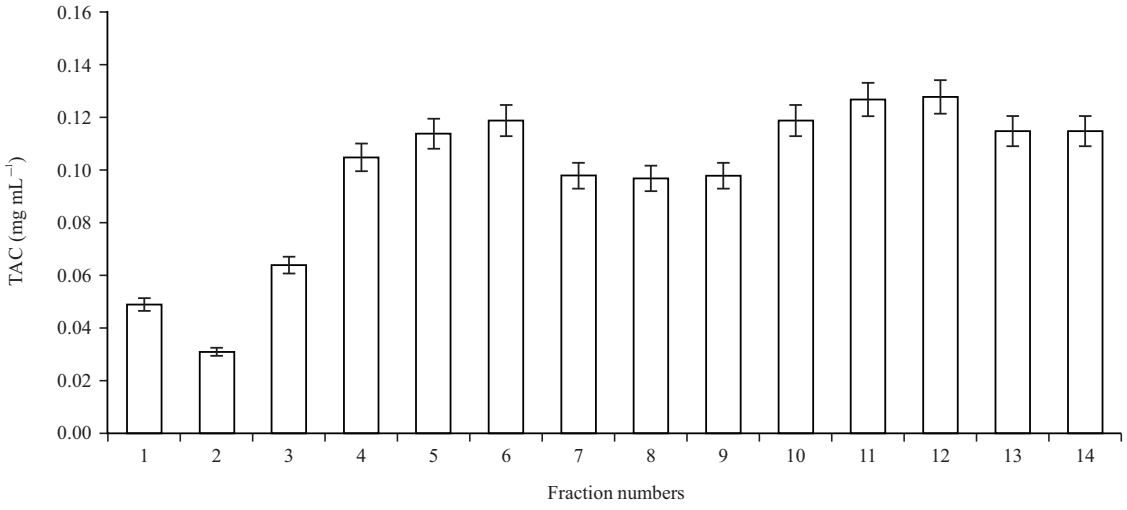

Fig. 1: Total antioxidant capacity (TAC) of different fractions of ethanolic extract of *D. oliveri* stem bark

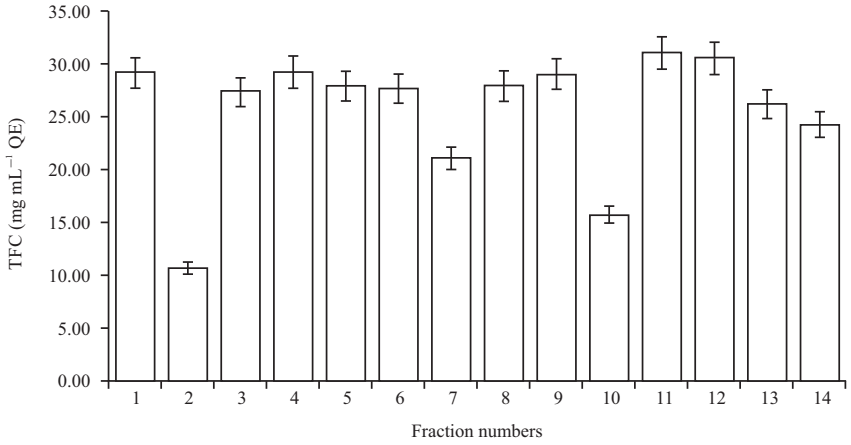

Fig. 2: Total flavonoids content (TFC) of different fractions of ethanolic extract of *D. oliveri* stem bark

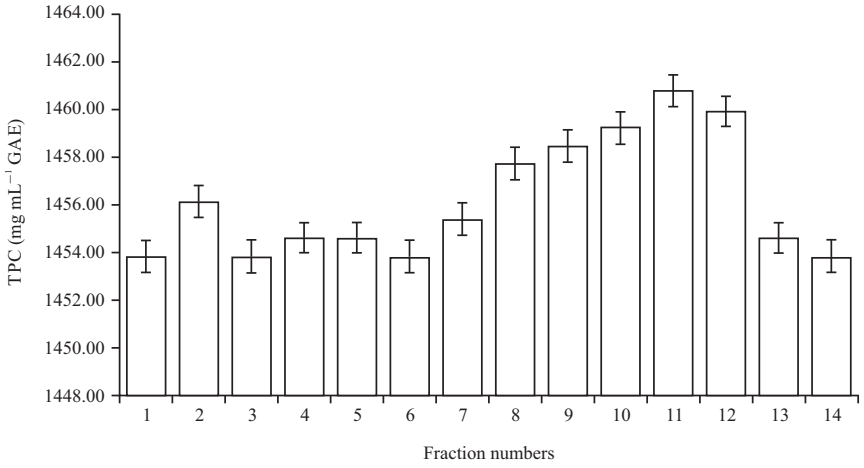

Fig. 3: Total phenolic content (TPC) of different fractions of ethanolic extract of *D. oliveri* stem bark

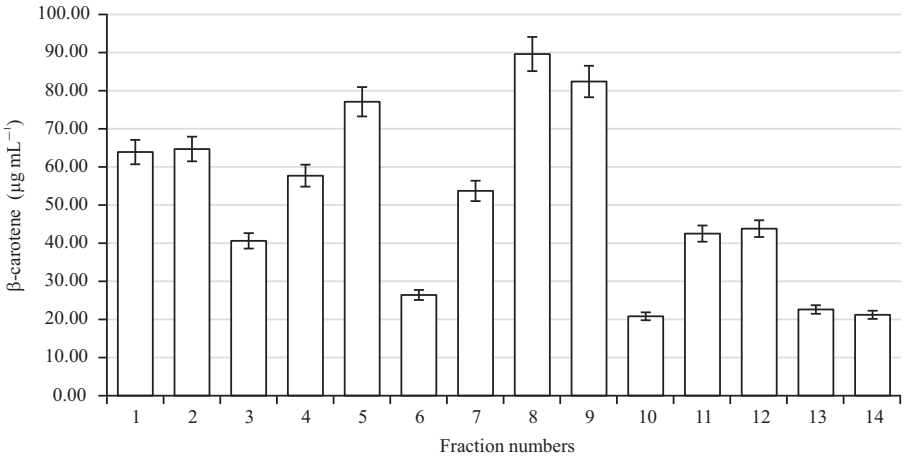

Fig. 4: β-carotene bleaching inhibition assay of different fractions of ethanolic extract of *D. oliveri* stem bark

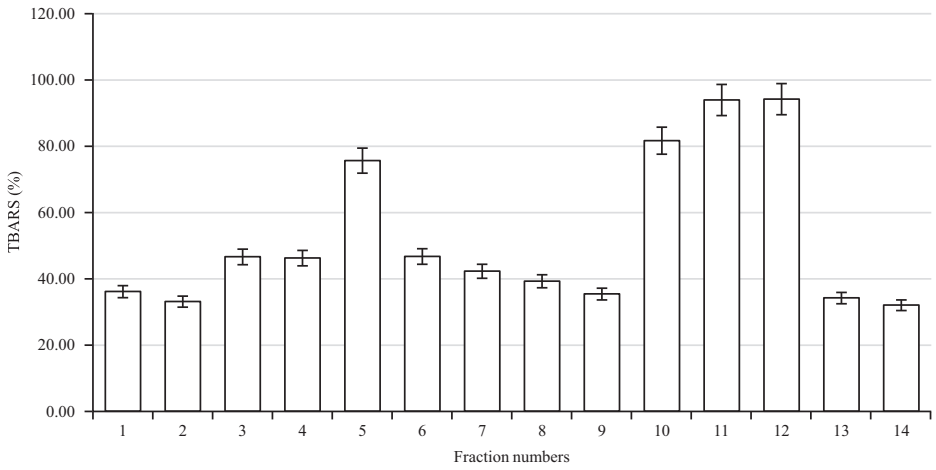

Fig. 5: Anti-lipid peroxidation effects of different fractions of ethanolic extract of *D. oliveri* stem bark

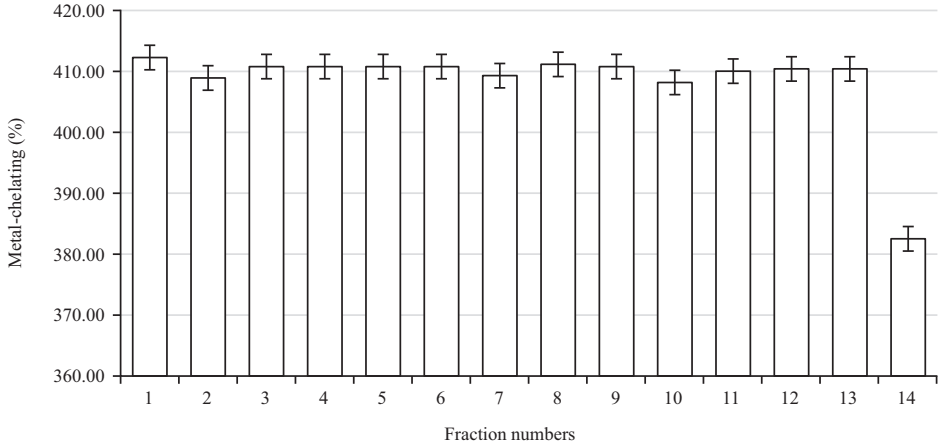

Fig. 6: Metal-chelating inhibition activity of different fractions of ethanolic extract of *D. oliveri* stem bark

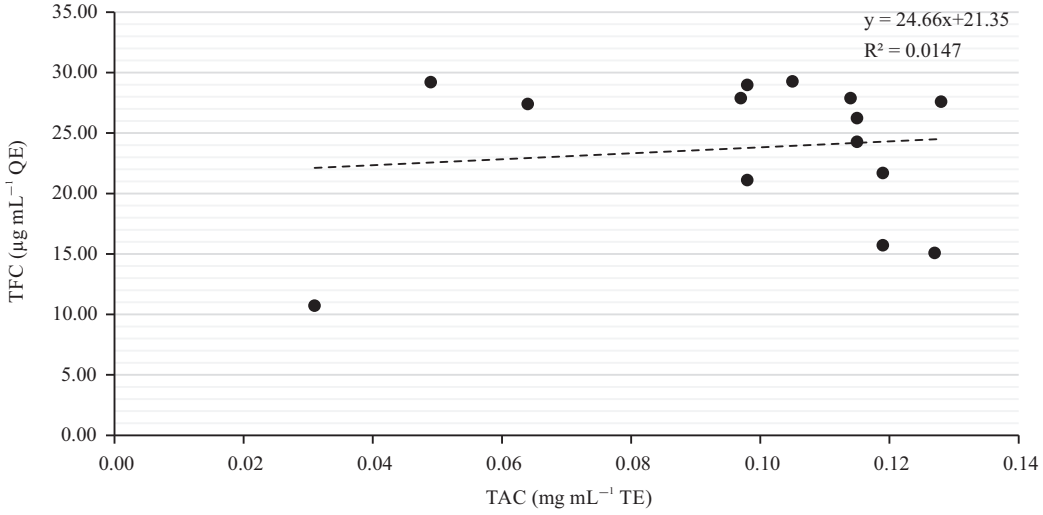

Fig. 7: Linear correlation between TAC/TFC of different fractions of ethanolic extract of *D. oliveri* stem bark

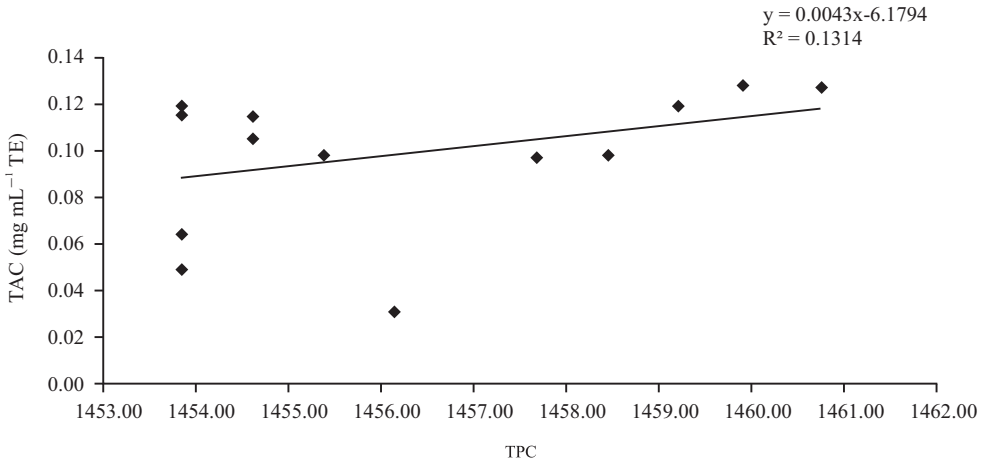

Fig. 8: Linear correlation between TAC/TPC of different fractions of ethanolic extract of *D. oliveri* stem bark

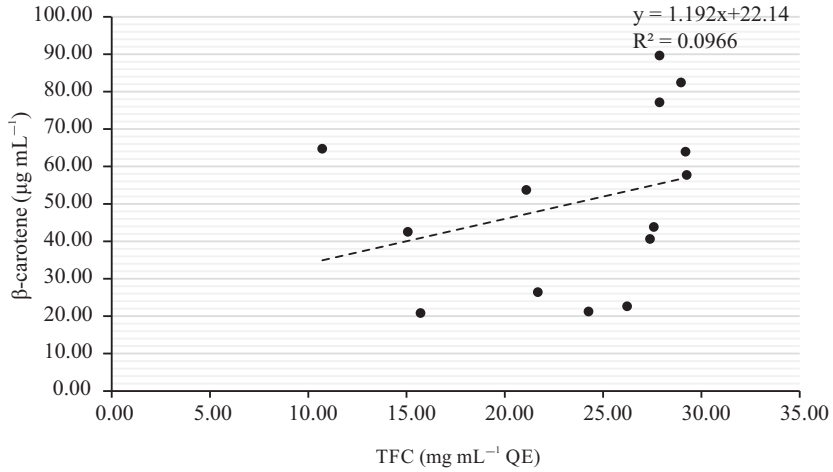

Fig. 9: Linear correlation between TAC/β-carotene bleaching inhibition of different fractions of ethanolic extract of *D. oliveri* stem bark

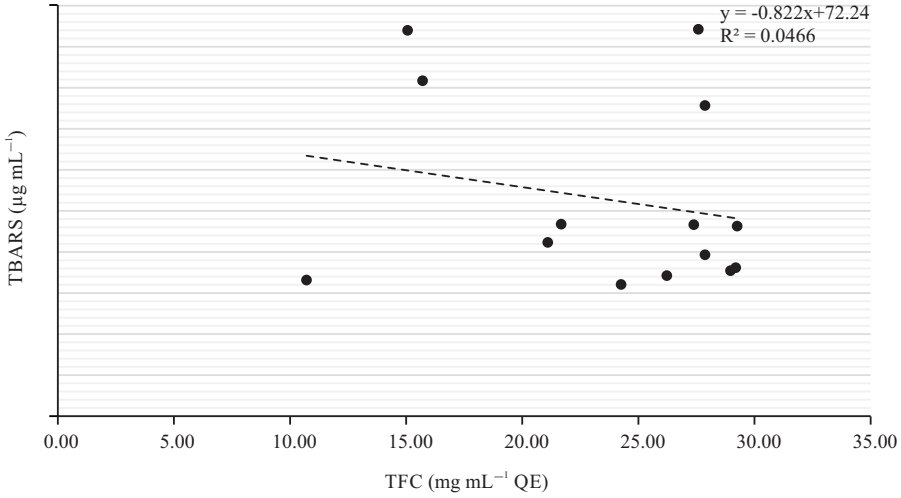

Fig. 10: Linear correlation between TFC/TBARS of different fractions of ethanolic extract of *D. oliveri* stem bark

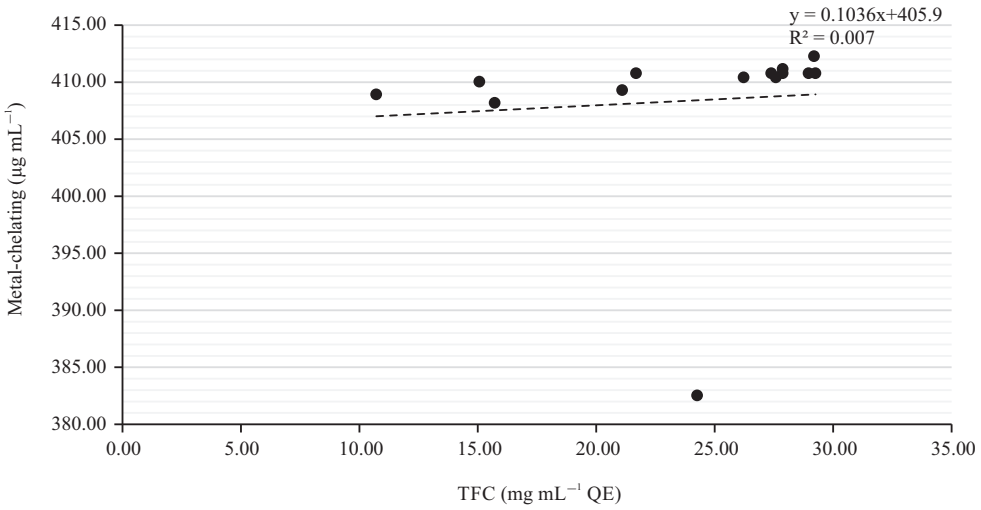

Fig. 11: Linear correlation between TFC/Metal chelating activity of different fractions of ethanolic extract of *D. oliveri* stem bark

This study agreed with Shabir *et al.*[25] that medicinal plants used in traditional healings have antioxidant properties. The results of antioxidant properties determined showed that the solvents used were all able to extract substances with antioxidants potentials. However, the ethyl acetate was less efficient in the extraction of these antioxidants substances compared to other employed solvents (Fig. 1). The reducing tendency of the extracts increased with ethanol:water fraction than other solvents and variations in reducing ability was observed among the different fractions considered (Fig. 1), suggesting that the solubility of the active principle relies greatly upon this particular solvent combination. This point to this fraction of the extract, among others for further scientific and industrial evaluation.

The total flavonoid content in the fractions ranged from 15.71-31.20 mg mL^{-1}, while that of total phenolics was between 1453.85 and 14460.77 mg mL^{-1} GAE (Fig. 2 and 3). That the phenolic content was much higher than the total flavonoid content, indicates that the plant contains much more phenolic compounds other than flavonoids. Reports by different researchers have established that most flavonoids have stronger radical-scavenging capacity due to their electron (hydrogen) releasing tendency[26] than other natural phenolic compounds[27-29].

The differences in the magnitude of effects displayed by flavonoids and phenolics from natural products could be a result of the type of plant, the exact chemical nature of the compound, the solubility of the different compounds and the effectiveness of extraction solvents in solubilizing such compounds[30,31]. In general, it has been suggested that solvents with moderate polarities are often preferred for the extraction of phenolics and antioxidant compounds as opposed to those with higher polarities like water or non-polar counterparts like hexane[32].

The β-carotene, a lipid-soluble compound of the terpenes subclass is a free radical scavenging antioxidant that is metabolized in the biological systems by some of the essential antioxidant enzymes that have antioxidant activity like superoxide dismutases[33]. In the absence of antioxidants, β-carotene forms hydroperoxides with resultant bleaching and change in colour as a result of linoleic acid oxidation. The fractions of ethanolic extract of *Daniellia oliveri* stem bark have been demonstrated in this study to show good antioxidant activity against the bleaching effect with increasing concentrations (Fig. 4), thus, suggesting the presence of antioxidant compounds that mitigates this bleaching effect in this assay[34].

Lipid peroxidation appears to be important in many human diseases including atherosclerosis, primary tissue damage and traumatic brain damage[35]. In addition, oxidative stress has deleterious effects on proteins and DNA which are even more significant targets of injury than lipids, but lipid peroxidation often is the last stage in the injury process. Thus, the application of simple diene-conjugate and Thiobarbituric Acid Reactive Substances (TBARS) assay to human tissues and body fluids has demonstrated considerable results of lipid peroxidation[36,37], suggesting lipid peroxidation as an important indicator of damaged tissues that are induced by reactive oxygen species[38,39]. Hence, the level of anti-lipid peroxidation effects shown by *D. oliveri* stems bark ethanolic extract and fractions (Fig. 5) indicates that these fractions possess anti-lipid peroxidation capacity in varying measures.

Iron, a metal, catalyzes important oxidation reactions and generates free hydroxyl radicals and peroxides, resulting in diseases. Thus, these oxidation reactions are delayed by iron chelators, which mobilize tissue iron by forming soluble and stable complexes. Ferrozine, an iron chelator quantitatively forms complexes with Fe^{2+}, where the presence of other chelating agents in extracts disrupts complex formation resulting in the formation of red color[40]. Measurement of colour reduction allows the estimation of the chelating activity of the existing chelator. Fe^{2+} (transition metal ion) possesses the ability to move single electrons and thus allow the formation and propagation of radical reactions. They mainly avoid reactive oxygen species (ROS) generation that is associated with redox-active metal catalysis involving the metal ion chelation. Fractions of ethanolic *Daniellia oliveri* stem bark extract contained antioxidant constituents that interfered with the formation of ferrous and ferrozine complexes (Fig. 6).

Correlation analysis showed positive but weak relationships between the results of TAC and TFC, TAC and TPC, TAC and β-carotene (Fig. 7-9) and an even weaker relationship between TBARS and between metal chelating assay and TFC (Fig. 9 and 10). The weak relationship exhibited by the fractions could signify that the elicited effects to a very large extent, are not dependent on the phenolic and flavonoid contents, although their presence suggests their contributions. This corroborates the findings of earlier workers[40].

These works are laboratory finding and have not been clinically proven. It is therefore, recommended for further scientific studies via structure elucidation of the active compounds and clinical validation of the claims.

CONCLUSION

Based on the data obtained in this study, it can be concluded that fractions of ethanolic extract of *D. oliveri* stem bark, particularly fractions 11 and 12, possess potent antioxidant activity by different methods, suggesting that this extract contains substances that could act through synergistic action related to, but not limited to direct free radical scavenging, chelation of transition metal and direct inhibition of lipid peroxidation.

SIGNIFICANCE STATEMENT

This study discovered the appropriate solvent for the extraction and purification of the active ingredient in this plant that can be beneficial for combating/management of oxidant-induced pathologies. This study will help the researchers to uncover the critical areas of this methodology that many researchers were not able to explore.

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