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Evaluation of Antioxidant Activity of *Thesium viride* Hill (Santalaceae)

Sani Shehu[1,2,*], Ezzeldin Mukhtar Abdurahman[2], Umar Habibu Danmalam[2], Abdullahi Mohammed[3], Aisha Ibrahim Barau[1]

[1]Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Kaduna State University, Kaduna, Nigeria

[2]Department of Pharmacognosy and Drug Development, Faculty of Pharmaceutical Sciences, Ahmadu Bello University Zaria, Nigeria.

[3]Department of Pathology, Faculty of Basic Clinical Science, College of Medicine, Ahmadu Bello University Zaria, Nigeria.

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* *Corresponding Author:*

pharmsani@gmail.com,
sani.shehu@kasu.edu.ng
https://orcid.org/0000-0002-4052-787
+2348036617718

ABSTRACT

Background: *Thesium viride* is commonly called 'Huntu' in Hausa. It is a hemi-parasite subshrub from the family Santalaceae. The aerial part of the plant is used in the treatment of jaundice, liver enlargement, splenomegaly and ulcers. Despite its uses in diseases that may be attributed to free radical there was no studies to evaluate of the antioxidant activity. The study aimed to evaluate the antioxidant activity of 70% aqueous ethanol extract of *T. viride* (AETV) and its fractions; ethyl acetate (ETV), butanol (BTV) and aqueous (ATV).

Methods: The plant was collected at Karaukarau, Giwa Local Government Area of Kaduna State and was identified and authenticated at the Herbarium Unit of the Department of Biological Sciences, Ahmadu Bello University. The aerial part of the plant collected was air dried under the shade and powdered. It was macerated with 70% aqueous ethanol and a portion of the extract was successively fractionated using ethyl acetate, n-butanol and water. The free radical scavenging activity of the 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), reducing power of ferricyanide ion (FRAP), total antioxidant capacity (TAC) of the phosphomolybdenum method and the estimation of total phenolics (TP) and total flavonoids (TF) contents have been determined for all studied extracts/fractions by spectrophotometric methods.

Results: Both TP (155.47 mg gallic acid equivalent [GAE]/g ± 10.32 mg GAE/g) and TF contents (103.29 mg quercetin equivalent [QE]/g ± 4.43 mg QE/g) were determined to be highest in ETV. The DPPH free radical, FRAP assays and TAC showed a concentration dependent activity with ETV exhibited the highest antioxidant capacity as determined by all the methods. Using Pearson's correlation analysis between the values of FRAP-TAC ($R^2 = -0.91$) at $p < 0.05$ indicates the viability of the two models for evaluating antioxidants from *T. viride*.

Conclusion: The antioxidant evaluation based on the models used in this study revealed that AETV and especially the ETV possess antioxidant activity.

1. Introduction

Free radicals are atoms or molecules that have unpaired electrons, usually unstable and highly reactive[1]. The two types of free radicals are oxygen-based radicals and nitrogen-based radicals. The oxygen free radicals, such as superoxide, hydroxyl radicals and peroxyl radicals, with the addition of non-radicals, such as hydrogen peroxide, hypochlorous acid and ozone are known as reactive oxygen species (ROS), which are generated during the metabolism process of oxygen. Reactive nitrogen species (RNS), including nitrogen-based radicals and non-radicals, such as nitrogen dioxide, nitric oxide radicals and peroxynitrite are derived from nitric oxide and superoxide via inducible

nitric oxide synthase (iNOS) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, respectively[2]. Both ROS and RNS are beneficial and toxic to the living system as they are involved in various physiological functions such as in immune function, cellular signaling pathways and mitogenic response and in redox regulation. Whilst at higher concentration they generate oxidative stress and nitrosative stress, respectively, causing potential damage to the biomolecules resulting to injury[3]. Free radicals are responsible for causing many diseases including cancer, cardiovascular disease, neural disorders, Alzheimer's disease, mild cognitive impairment, Parkinson's disease, alcohol induced liver disease, ulcerative colitis, ageing and atherosclerosis[4].

Antioxidants are compounds that can scavenge, dispose and prevent the generation of free radicals or challenge their actions[5]. There is growing interest nowadays to plant materials that have antioxidant properties containing wide range of compounds that possess antioxidant properties able to capture free radicals. These includes carotenoids, phenolics, flavonic, anthocyanic derivatives, unsaturated fatty acids, vitamins, enzymes and cofactors which stimulate interest in using them in prophylactic and curative phytotherapy[6].

Thesium viride is also known as *T. hararensis* A.G. Mill and *T. unyikense* Engl.[7]and commonly called 'Huntu' in Hausa in Northern Nigeria[8]. It is a hemiparasite subshrub with tufts of stems arising from a woody rootstock with alternate, simple acuminate apex leaves. The aerial part of the plant is used in the treatment of jaundice, liver enlargement, splenomegaly, and cure ulcers[9,10]. The aqueous ethanol extract of the plant contains alkaloids, flavonoids, cardiac glycosides, anthraquinones and other phenolic compounds[8]. Kaempferide was isolated from the ethyl acetate fraction of aqueous ethanol extract of the plant[11]. The aqueous ethanol extract was found to be active against *Staphylococcus aureus, Streptococcus faecalis, Escherichia coli, Helicobacter pylori* and *Shigella dysenteriae*[8].

Despite the use of this plant to treat conditions associated with oxidative stress, there is no report in the literature for the evaluation of the antioxidant activity of this plant. Therefore, the present study was undertaken to evaluate the antioxidant potential by using different assays.

2. Materials and methods

2.1 Materials

List of reagents used

1, 1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma-Aldrich Co.), anhydrous sodium carbonate, Ascorbic acid, Folin-Ciocalteu phenol reagent, gallic acid, quercetin (Fluka, UK), and all other chemicals were of analytical grade were obtained from Merck (Darmstadt, Germany).

2.2 Methods

2.2.1 Collection and preparation of plant materials

The plant was collected at Karaukarau, Giwa Local Government Area of Kaduna State (11 °07´40´´N 7 °30´04´´E) and it was identified and authenticated at the Herbarium Unit of the Department of Biological Sciences, Ahmadu Bello University, Zaria with voucher number 90000415. The aerial part of the plant collected was air dried under the shade and powdered.

The dried powdered aerial part of the plant (1 kg) was extracted with 2 L of aqueous ethanol (70%v/v) in a glass jar for 7 days at room temperature with gradual agitation and change of solvent after 48 h. The combined extract was filtered through Whatman filter paper and concentrated on a Buchi rotary evaporator at 50°C with a reduced pressure. The aqueous ethanol extract (40 g) was suspended in 200 mL distilled water and filtered through Whatman filter paper size 2. The filtrate was transferred into a separating funnel and partitioned twice with 100 mL of ethyl acetate. The aqueous portion was then partitioned twice with 100 mL of saturated n-butanol on equilibration. The partitions ethyl acetate (ETV), n-butanol (BTV) and residual aqueous (ATV) were concentrated and dried.

2.2.2 Phytochemical screening

Phytochemical screening to detect the presence of plant constituent was performed by standard procedure described by Evans[12]. After the addition of some specific reagents to the solution, the tests were detected by visual observation of colour change or by formation of precipitate.

2.2.3 Determination of total phenolic content

The total phenolic (TP) content of the 70% aqueous ethanol extract and fractions of *T. viride* were determined by using Folin–Ciocalteu reagent (diluted 1:10 with deionised water) as described by Alhakmani et al[13]. Gallic acid was used as a reference standard for plotting calibration curve. A volume of 0.5 mL of extract (100 µg/mL) was mixed with 2 mL of the Folin–Ciocalteu reagent and neutralised with 4 mL of 7.5%, w/v sodium carbonate solution. The reaction mixture was incubated for 30 min at room temperature with intermittent shaking for colour development. The absorbance of the blue colour formed was measured at 765 nm using spectrophotometer (Spectrumlab 23A). The TP

contents were determined from the linear equation of a standard curve. The content of TP compounds expressed as mg/g garlic acid equivalent (GAE) of dry extract. All the determinations were carried out in triplicate[13]. The TP contents were calculated using the following linear equation based on the calibration curve of gallic acid:

$A = 0.0548x - 0.0036$ ($R^2 = 0.9925$)……..Equation 1

Where A is absorbance and x is amount of gallic acid in microgram.

2.2.4 Determination of total flavonoids content

Aluminium chloride colorimetric method was used for the determination of the total flavonoid (TF) content of the sample as described by Chandra et al.[14]. The standard calibration curve was made using quercetin. Stock solution of quercetin was prepared by dissolving 5 mg quercetin in 1.0 mL methanol, then the standard solutions of quercetin were prepared by serial dilutions using methanol (0.2 mg/mL – 1 mg/mL). An amount of 0.6 mL diluted standard quercetin solutions or extracts and fractions were separately mixed with 0.6 mL of 2% aluminium chloride. The mixed solution was incubated at room temperature for 60 min. The absorbance of the reacted mixtures were measured against blank at 420 nm wavelength. The concentration of TF content in the test samples was calculated from the calibration plot and expressed as mg quercetin equivalent (QE)/g of dried plant material. All the determinations were carried out in triplicate. The TF contents were calculated using the following linear equation based on the calibration curve:

$A = 0.9575x + 0.0161$, ($R^2 = 0.9935$). Where A is absorbance and x is amount of quercetin in microgram.

2.2.5 *In vitro* antioxidant activities of extract and fractions of *T. viride*

Evaluation of free radical scavenging activity

The 1-diphenyl-2-picrylhydrazyl (DPPH) assay reported by Cobaleda-Velasco et al.[15] was used to evaluate the free radical scavenging activity. Five concentrations of the extract and each fraction (6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL) was prepared and individually analysed. Extract (1 mL) was added to 3 mL of 0.1 mM DPPH (prepared in ethanol) and allowed to stand for 30 min at room temperature and darkness. Then absorbance at 523 nm was read. The ability of the scavenging effect was calculated as:

$$\text{Radical Scavenging Activity} = \frac{(\text{Absorbance of Control} - \text{Absorbance of test sample})}{(\text{Absorbance of Control})} \times 100 \ldots\text{Equation 2}$$

Evaluation of ferric reducing power

The reduction potential of plant extract and fractions were measured by the ability to reduce ferricyanide, ion that is, $[Fe(CN)^6]^{3-}$ to ferrocyanide ion, that is, $[Fe(CN)^6]^{4-}$ as described by Arvinder *et al*[16] with slight modification. The extract/ fractions (0.75 mL) at various concentrations (0 µg/mL, 6.25 µg/mL, 12.5 µg/mL, 25 µg/mL, 50 µg/mL, 100 µg/mL) was mixed with 0.75 mL of phosphate buffer (0.2 M, pH 6.6) and 0.75 mL of potassium hexacyanoferrate ($K_3Fe(CN)^6$) (1%, w/v), followed by incubation at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75 mL of 10% trichloroacetic acid solution then centrifuged at 3000 rpm for 10 min. 1.5 mL of the supernatant was mixed with equal volume of distilled water and 0.1 mL of 0.1%, w/v ferric chloride solution was added and kept at room temperature for 10 min. The absorbance was taken at 700 nm. The high absorbance of the reaction mixture indicates greater reducing power.

Evaluation of total antioxidant capacity

The total antioxidant capacity (TAC) of the 70% aqueous ethanol extract and various fractions were evaluated by the phosphor-molybdenum method according to the procedure described by Prieto *et al*[17]. The assay is based on the reduction of Mo (VI) to Mo (V) by plant extract and subsequent formation of green color of Mo (V) phosphate complex at an acidic pH. A volume of 0.3 mL extract was combined with 3 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The absorbance of the reaction mixture was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol was placed as the blank instead of the extract. The antioxidant activity is expressed as gram equivalent of ascorbic acid. The calibration curve was prepared by mixing ascorbic (1000 µg/ mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL and 31.25 µg/mL) with methanol.

2.6 Statistical analysis

Mean, standard error and correlation coefficients were determined amongst DPPH, reducing power of ferricyanide ion (FRAP) assay, TAC assay, total phenol and flavonoid contents using Microsoft Excel software 2013.

3 Results

3.1 Phytochemical screening

The results of phytochemical screening of 70% aqueous ethanol and fractions of *T. viride* are given in Table 1. The extract, phytochemical analysis revealed the presence of flavonoids, tannins, steroid/triterpenes and cardiac

glycosides. Likewise, the fractions contain these phytochemicals except for cardiac glycoside and alkaloids in ethyl acetate and aqueous fractions, respectively. Anthraquinones were not detected in all extract and fractions.

Table 1: Phytochemicals detected in extract and fractions of *T. viride*

Phytochemical	Aqueous ethanol extract	Ethyl acetate fraction	Butanol fraction	Aqueous fraction
Steroid/Triterpenes	+	+	+	+
Flavonoids	+	+	+	+
Tannins	+	+	+	+
Anthraquinone	-	-	-	-
Alkaloids	+	+	+	-
Cardiac Glycoside	+	-	+	+

3.2 Total phenols and flavonoids content of *T. viride* extract and fractions

The TP content in the extract and different fractions are shown in Table 2. The maximum amount of phenolic content was found in ethyl acetate fraction (155.48 mg.GAE/g) followed by n-butanol fraction with 129.93 mg.GAE/g. The 70% aqueous ethanol extract and aqueous fraction at 106.21 mg.GAE/g and 73.36 mg.GAE/g, respectively. The TFs content in the extract and different fractions are shown in Table 2. The highest amount of flavonoid content was found in ethyl acetate fraction (103.29 mg.QE/g) followed by the 70% aqueous ethanol extract, butanol and aqueous fraction at 87.62 mg.QE/g, 79.79 mg.QE/g and 18.17 mg.QE/g, respectively.

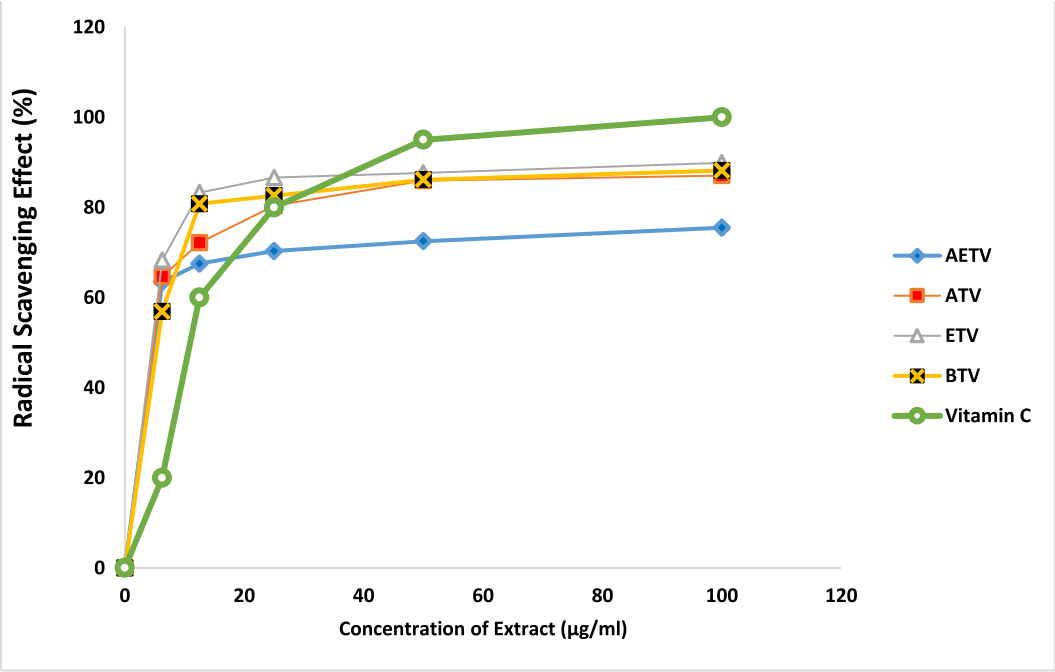
Table 2: Total phenols and flavonoids of the extract and fractions of *T. viride*

Extract/Fractions	Total Phenols (mg.GAE/g)	Total Flavonoids (mg QE/g)
AETV	106.20 ± 2.58	87.62 ± 2.95
ETV	155.47 ± 10.32	103.29 ± 4.43
BTV	129.93 ± 5.16	79.79 ± 3.69
ATV	73.36 ± 2.58	18.17 ± 3.69

AETV: Aqueous ethanol extract of *T. viride*, ETV: Ethyl acetate fraction of *T. viride,* BTV: butanol fraction of *T. viride,* ATV: Aqueous fraction of *T. viride*, GAE: Gallic acid equivalent, QE: Quercetin equivalent

3.3 Radical scavenging activity of *T. viride* extract and fractions

Free radical scavenging activity of *T. viride* and the reference standard (ascorbic acid) were evaluated using DPPH assays. The results are presented in Figure 1. All the samples exhibited free radical scavenging activity expressed in terms of percentage. The DPPH radical scavenging capacity of ascorbic acid at 6.25 μg/mL and 100 μg/mL were 25% and 100%, respectively. A high free radical scavenging activity is exhibited by the ethyl acetate fraction of 68.33% and 89.99% at 6.25 μg/mL and 100 μg/mL, respectively. The activity increases with increase in concentration in the extract and fractions.

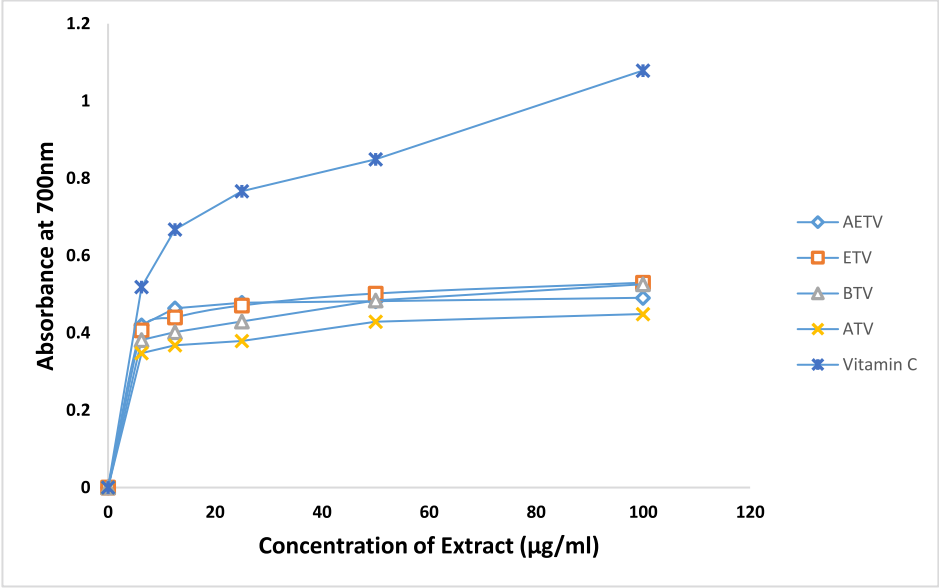


AETV: Aqueous ethanol extract of *T. viride*, ETV: Ethyl acetate fraction of *T. viride,* BTV: Butanol fraction of *T. viride,* ATV: Aqueous fraction of *T. viride*.

Figure 1: DPPH radical scavenging activity of 70% aqueous extract and fractions of *T. viride.*

3.4 Reducing power of *T. viride* extract and fractions

The extract and fractions reduces the Fe^{3+} in a concentration-dependent manner in the FRAP assay. Reducing power of substance increases accordingly to the increase in absorbance as shown in ascorbic acid in Figure 2. The extract and fractions showed increase reduction power with increasing concentration. The reducing power of the *T. viride* was found remarkably lower than ascorbic acid.

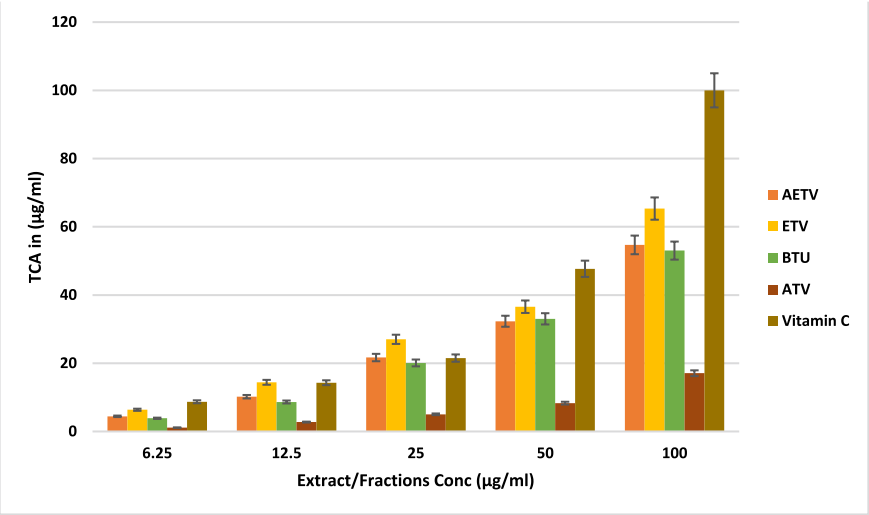


AETV: Aqueous ethanol extract of *T. viride*, ETV: Ethyl acetate fraction of *T. viride,* BTV: Butanol fraction of *T. viride,* ATV: Aqueous fraction of *T. viride*,

Figure 2: Reducing power of 70% aqueous extract and fractions of *T. viride.*

3.5 Total antioxidant capacity of *T. viride* extract and fractions

The result of total antioxidant activity (TAC) is shown in Figure 3. Ethyl acetate fraction possesses significant TAC equivalent to 65.34 mg/g ascorbic acid at the highest concentration of 100 µg/mL, which is followed by 70% aqueous extract 54.69 mg/g ascorbic acid. Also, n-butanol fraction and aqueous fraction has 53.03 mg/g and 17.07 mg/g ascorbic acid.



AETV: Aqueous ethanol extract of *T. viride*, ETV: Ethyl acetate fraction of *T. viride,* BTV: Butanol fraction of *T. viride,* ATV: Aqueous fraction of *T. viride*,

Figure 3: Total antioxidant capacity of 70% aqueous ethanol extract and its fractions.

3.6 Correlation of assays

The results revealed different correlations between the antioxidant activities (DPPH, FRAP and TAC) and their contents (TP and TF) as shown in table 3. The strongest correlation within the antioxidant activity assay was observed in FRAP-TAC (R^2 = -0.91). Moderate correlation was observed in DPPH-FRAP (R^2=-0.43) while a weak correlation in DPPH-TAC (R^2=0.28). There is also a strong correlation between the total phenol and flavonoid content (R^2= 0.88) with an excellent correlation between the total antioxidant capacity and total flavonoids (R^2= 0.99).

Table 3: Correlation between the different assays used in this study.

Correlation	R^2
DPPH-FRAP	-0.43
DPPH-TAC	0.28
DPPH-TP	0.35
DPPH-TF	-0.13
FRAP-TAC	-0.91*
FRAP-TP	-0.97*
FRAP-TF	0.87
TAC-TP	0.91
TAC-TF	0.99**
TP-TF	0.88

Pearson' Correlation Coefficient * $p < 0.05$ and ** $p < 0.01$

4. Discussion

Phenolics and flavonoids in general, constitute a major group of compounds, which act as primary antioxidants, and are known to react with hydroxyl radicals, superoxide anion radicals and lipid peroxy radicals[14]. Studies reveal that the antioxidant activity of phenolic compounds found in fruits, vegetables and herbs are attributed to their redox properties allowing them to act as reducing agents, singlet oxygen quencher, hydrogen donators and chelating agents of metal ions[16]. A significant relationship between antioxidant potential and phenolic content in *T. viride* might be the major contributors to the antioxidant potential of the extract and fractions.

The DPPH, FRAP and TAC assays were used to measure the antioxidant activity of the plant. This is because different antioxidant compounds may act *in vivo* through different mechanisms as no single method can fully evaluate the antioxidant capacity of a substance. Therefore, it is essential to use more than one method to evaluate antioxidant capacity of plant materials because of the complex nature of phytochemicals[18]. In DPPH assay, a high free radical scavenging activity is exhibited by the ethyl acetate fraction which increases with increase in concentration. Likewise, a promising radical scavenging activity was observed in n-butanol and aqueous fractions better than extract. The extract had the steady linear free radical scavenging activity against the concentration. The high concentration of phenolic and flavonoids in this fraction contributed to the scavenging effect. The extract and fractions reduce the Fe^{3+} in a concentration-dependent manner in the FRAP assay. The reducing power of the *T. viride* was found remarkably lower than the standard (ascorbic acid). The ethyl acetate fraction shows a better reducing ability than butanol fraction, extract and aqueous fraction at 50 µg/mL. The activity at the highest concentration was found in the order ETV>BTV> AETV>ATV. The TAC is based on the reduction of Mo (VI) to Mo (V) by the extract/fractions at acidic pH. This model evaluates both water-soluble and fat-soluble antioxidant potential[20]. It was observed that the ethyl acetate fraction possesses significant TAC equivalent to 65.34 mg/g ascorbic acid at the highest concentration of 100 µg/mL. The results indicate a concentration dependent TAC meaning that the extract or fractions will have to contain as much quantity of antioxidants substances as equivalents of ascorbic acid to effectively reduce the oxidant substance in the reaction system.

Ethyl acetate fraction was found to have the highest antioxidant capacity, which can also be attributed to the phenols ($R^2 = 0.91$) and flavonoids ($R^2 = 0.99$) compounds present[20]. Phenolics and flavonoids, in general, constitute a major group of compounds, which act as primary antioxidants, and are known to react with hydroxyl radicals, superoxide anion radicals and lipid peroxy radicals[18]. A significant relationship between antioxidant potential and total phenol content was found, indicating that phenolic compounds might be the major contributors to the antioxidant potential.

In this study, the findings show that phenolic content was better correlated with both FRAP ($R^2 = -0.97$) and TAC ($R^2 = 0.91$) assays than with that of DPPH ($R^2 = 0.35$) as shown in table 3. These results suggest that 97% of the ferric reducing power and 91% of the ability to reduce Mo (VI) to Mo (V) ion is probably because of phenolic compounds. Also, it can be concluded that antioxidant activity of plant extracts is not limited to only phenolics. Activity may also be because of the presence of other secondary metabolites, which are known to be antioxidants such as volatile oils, carotenoids and vitamins. These non-phenolic compounds appear to be more important in contributing to activity in the DPPH assay. The antioxidant activity of phenolics is mainly because of the ease to act as reducing agents, hydrogen donors and singlet oxygen quenchers.

Flavonoids are group of phenolics, derived from tyrosine, phenylalanine and malonate and are well known for their antioxidant activities and over-accumulation in plant could enhance oxidative and drought tolerance phenomenon[21]. Flavonoid may have antioxidant effect as hydrogen-donating compound, metal chelating ion, single oxygen transfer and singlet oxygen quencher[21]. Ethyl acetate fraction had the highest whilst aqueous fraction had the least flavonoid content. The results revealed excellent correlations between the antioxidant assays and flavonoid content in FRAP ($R^2 = 0.87$) and TAC ($R^2 = 0.99$) assays. The correlation coefficients that were determined indicate that the antioxidant activities by these two methods possessed by the extract/fractions may be principally caused by the flavonoid compounds present. On the other hand, an inferior correlation was observed between the flavonoid contents and the antioxidant activities of the extract/fractions in DPPH ($R^2 = -0.13$). The antioxidant activity may likely not be as a result of the presence of flavonoids in the extract and fractions but other phenolics and to larger extent other secondary metabolite in the extract and fractions.

Based on Pearson's correlation in table 3, the strongest correlation was found between FRAP and TAC whilst moderate with DPPH. Also, antioxidant capacity using

FRAP has a strong negative correlation with the total phenol contents. These results suggest that the extract and fractions reducing power may be associated with the phenolic compounds or rather other compounds with hydrogen donating ability.

5. Conclusion

The results of antioxidant evaluation based on the models used in this study revealed that the 70% aqueous ethanol extract of *T. viride* and especially the ethyl acetate fraction possess interesting antioxidant activity. The correlation analysis between the antioxidant study activity models and the total flavonoids content indicates the possibility that flavonoids are the major constituents in the plant responsible for the antioxidant activity. This finding established the potentials of *T. viride* as important source of natural antioxidants which may have been the basis for its use in treatment of illness associated with oxidative stress.

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Competing interests

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