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Research Article

Phytochemical Composition, Antioxidant and Antitrypanosomal Properties of Leaves and Stem Bark Extract of *Detarium microcarpum*: An *in vitro* study

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ABSTRACT

Trypanosomiasis is a major disease in the Africa continent that affects both humans and livestock. This study investigated the phytochemical composition, antioxidant and antitrypanosomal activities of the extracts from Detarium microcarpum leaves and stem bark. Sequential extraction of the plant samples was done with the solvents of increasing polarity and these extracts were assayed for antioxidant properties, phytochemical constituents, and antitrypanosomal (*Trypanosoma congolense* and *Trypanosoma brucei brucei*) activity using standard protocols. The result from the phytochemical studies showed that methanolic extract of the stem bark has the highest flavonoid and phenolic contents (596.88 ± 0.13 mgQE/mg of extract and 367.3 ± 15.84 mg/GAE/mg of extract). The *in vitro* antioxidant study showed that there was no significant difference in the DPPH scavenging activities of the various extracts compared to ascorbic acid. However, aqueous stem bark extract and methanolic extracts of the leaves and stem bark showed a significant difference in H_2O_2 scavenging activity when compared to ascorbic acid, with an IC_{50} value of 1227.04 ± 77.19 µg/mL, 109.61 ± 13.38 µg/mL and 447.40 ± 12.04 µg/mL. From the in vitro antitrypanosomal studies, methanolic leaves extract of D. microcarpum showed an efficient antitrypanosomal effect with complete cessation in motility at 5 min against *Trypanosoma congolense*, and at 10 min against *Trypanosoma brucei brucei* at the highest concentration of the extract (20 mg/mL). The result of this study shows that the various extracts of *D. microcarpum* possess possible inhibitory potential against free radicals in addition to its antitrypanosomal effect.

Keywords: *Phytochemical, Detarium microcarpum, oxidative stress, Trypanosoma congolense, Trypanosoma brucei brucei*

INTRODUCTION

Animal African trypanosomosis (AAT) is a vector-borne disease of livestock caused by trypanosomes. The organisms that cause AAT have been found in many species of mammals, including domesticated animals and some wildlife. Cattles are reservoir for *Trypanosoma congolense*, *Trypanosoma vivax* and *Trypanosoma brucei brucei*, but other animals including small ruminants, pigs and some wildlife (e.g. African buffalo) are also thought to maintain these organisms. *T. b. brucei* is limited in its distribution, although it is not human infective due to its susceptibility to lysis by trypanosome lytic factor-1 (TLF-1). Its infection has been reported in humans lacking the TLF-1 (Deborgraeve *et al.*, 2008). *T. congolense* is a parasite responsible for the severe disease of African livestock. Its life cycle is complex and divided into two phases: one in the tsetse fly vector and the other in the bloodstream of the mammalian host (Takeet

et al., 2013; Mamoudou *et al.,* 2016). *T. congolense*, *T. evansi*, *T.vivax*, and *T. b. brucei* are the major agents of trypanosomiasis in livestock. The inability of the immune systems of livestock to deal with these trypanosomes is a major cause of the manifestation of the disease (Cross *et al.,* 2014; Umar *et al.,* 2018). Major symptoms of this disease include fever, severe anemia, cachexia, edema, and reproductive disorders that can lead to death if untreated (Balogun *et al.,* 2019).

Trypanosomiasis is responsible for the death of 3 million heads of cattle yearly, with 50 million animals at risk in sub-Saharan Africa (Chitanga *et al.,* 2011). The annual estimated direct and indirect losses due to the disease run into billions of dollars (Simarro *et al.,* 2012; Mulenga *et al.,* 2020). The existing treatments (suramin, melarsoprol, eflornithine and nifurtimox) of trypanosomiasis are challenged with problems comprising drug resistance, toxicity, high cost, and limited availability (Umeakuana *et al.,* 2019). Unfortunately, the high incidence of side effects and development of resistant strain has rendered existing chemotherapy inadequate. Increased oxidative stress is an established feature of AAT and it has been shown that reduction of this stress by antioxidants aids the host to keep the parasitemia low, thereby ameliorating the disease condition. Nature with its abundant plant species is a potential source of new drug since it contains numerous molecules with a great variety of pharmacological activities (Ali *et al.,* 2018). The diversity of natural products derived from plants have been observed to serve as potential source to obtain new drug target that maybe active against trypanosomes (Feyera *et al.,* 2014). *D. microcarpum* has been reported in scientific literature to possess medicinal properties and in view of this, there is a continuous search for acceptable, less expensive and nontoxic trypanocides. This study is aimed at investigating the *in vitro* antioxidant and antitrypanosomal activities of *Detarium microcarpum* leaves and stem bark extracts.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals and reagents used were of analytical grade and they include; Ethylene diamine tetraacetic acid (EDTA), Phosphate buffer saline (PBS). Methanol, n-hexane, Dimethyl sulfoxide (DMSO), Glacial acetic acid, 0.02M Ferric chloride, Sulfuric acid, Chloroform, ammonia, HCl, Lead sub-acetate, gallic acid, Quercetin, 10% Aluminium chloride, 1M Na_2CO_3, distilled water.

Plant Collection and Identification

The leaves and stem bark of *Detarium microcarpum* were collected at Basawa, Sabon Gari LGA of Kaduna State Nigeria and were identified by a qualified taxonomist at the Herbarium unit of the Department of Botany, Faculty of Life Sciences, Ahmadu Bello University, Zaria, Kaduna state, Nigeria where a voucher specimen was deposited and assigned a number V/N 90028.

Test Organisms

T. congolense and *T b. brucei* were obtained from stabilate maintained at the animal house of the faculty of pharmaceutical sciences Ahmadu Bello University Zaria. The molecular characterization of the parasite was carried out at the Africa Centre of Excellence for Neglected Tropical Disease and Forensic Biotechnology A.B.U Zaria and confirmed to be *T. congolense* and *T b. brucei*. The parasites were maintained in the laboratory by a continuous passage in rats until required. The passage was considered necessary when parasitemia was in the range of 16–32 parasites per field.

Preparation of Extract

The leaves and stem bark of the plant were washed with clean water and shed dried at room temperature for three weeks. After which they were milled separately using the Thomas-WILEY laboratory mill model 4. Exactly 600g of the powdered leaves and stem bark were weighed and exhaustively extracted by cold maceration for 72 hours using 1500 mL of different solvents of increasing polarity (distilled water, n-hexane, and methanol). The mixtures of the different extracts were passed through a mesh sieve (1mm), filtered using Whatman filter paper no. 1. The filtrates were concentrated to dryness using rotary evaporator at 45^{o}C. The extracts were kept in sample bottles and stored in the refrigerator at 4 ^{o}C.

Qualitative Phytochemical Screening

The phytochemical screening of the extracts was carried out using the method of Trease and Evans (1983) with slight modification. Alkaloids, glycosides, tannins, flavonoids, saponins, terpenoids, polyphenols, and steroids were screened.

Determination of Total Phenolic Content (TPC)

The total phenolic content of the extracts was estimated using the Folin-Ciocalteu reagent following the method of Alhakmani *et al*., (2013) with slight modification. Aliquot of 0.5 mL of the plant extract (100 µg/mL) was mixed with 2mL of Folin-ciocalteu reagent (1:10 in deionized water) and neutralized with 4 mL of saturated sodium carbonate

solution (7.5% w/v). The tubes were covered with foil paper and incubated at room temperature for 30mins with intermittent shaking. The absorbance was taken at 765nm using methanol as blank, all the samples were analyzed in duplicates. The total phenol was determined with the help of a standard curve prepared from a pure phenolic standard (Gallic acid).

Determination of Total Flavonoid content (TFC)

The total flavonoid content of the extracts was estimated using the method of Zhishene *et al.,* (1999) with slight modification. 0.5 mL aliquot of the sample and standard solution (0.01 – 1.0 mg/mL) of quercetin were mixed with 2 mL of distilled water. Subsequently, 0.15 mL of sodium nitrite (5% $NaNO_2$, w/v) solution was added. After 6mins, 0.15mL of 10% $AlCl_3$ w/v solution was then added. The solutions were allowed to stand for 6mins and 2 mL of sodium hydroxide (4% NaOH, w/v) solution was added to the mixture and allowed to stand for another 15 mins. The absorbance of the extracts was taken at 510 nm, TFC was determined as mg quercetin equivalent per gram of sample with the help of calibration curve of quercetin. All determinations were done in duplicates.

***In vitro* Antioxidant Studies**

DPPH Scavenging Activity

The DPPH free radical scavenging ability of the extracts was performed using the method of Liyana and Shahidi (2005) with slight modification. A solution of DPPH was prepared by dissolving 13.5mg in 100mL of 70% methanol. 2.0 mL of the DPPH solution (0.135mM) was added to 2 mL of various concentrations of the extracts (10-50 µg/mL) prepared in methanol. The reaction mixtures were vortexed and left in the dark at room temperature for 30mins. The absorbance of the mixtures was measured spectrophotometrically at 517nm. The ability of the plant extract to scavenge DPPH radical was calculated by the equation:

DPPH radical scavenging activity =

$$\{(Abs_{control} - Abs_{sample})/ Abs_{control}\} \times 100$$

Where; $Abs_{control}$ is the absorbance of DPPH radical + methanol
Abs_{sample} is the absorbance of DPPH radical + sample extract or standard

Hydrogen Peroxide Scavenging Activity

The hydrogen peroxide free radicals scavenging ability of the extracts was performed using the method of Umar *et al.*, (2018) with slight modification. 1mL of 0.1 mM H_2O_2 was mixed with 1mL of various concentrations (100, 200, 300, 400, 500 µg/mL) of the extracts, followed by the addition of 100 µL of 3% ammonium molybdate, 10 mL sulfuric acid (H_2SO_4) (2 M), and 7.0 mL KI (1.8 M). The solution was then titrated with sodium thiosulfate ($Na_2S_2O_3$) (5 mM) until the disappearance of the yellow color.

The percentage scavenging activity of the extract was calculated as;

H_2O_2 radical scavenging activity (%) =

$$\{(V_0 - V_1/V_0)\} \times 100\%$$

Where: V_0 is the volume of $Na_2S_2O_3$ solution used to titrate the control in the presence of H_2O_2 (without the extract), While V_1 is the volume of $Na_2S_2O_3$ solution used in the presence of the extract.

Determination of Parasitemia

Parasitemia was monitored in blood obtained from the tail of the donor rat, pre-sterilized with 70% ethanol, and the number of parasites was determined microscopically at ×400 magnification using the "Rapid Matching" method of Herbert and Lumsden (1976). The method involves microscopic counting of parasites per field in pure blood or blood appropriately diluted with buffered phosphate saline (PBS, pH 7.4). Logarithm values of these counts obtained by matching with the table of Herbert and Lumsden (1976) are converted to antilog to provide an absolute number of trypanosomes per ml of blood.

In Vitro* Antitrypanosomal Activity of *Detarium microcapum* Leaves and Stem Bark Extracts Against *T. b brucei* and *T. congolense.

The method of Atawodi *et al*., (2003) was adopted with slight modification. Exactly 10 mg of different solvent extracts of the plant were weighed and first dissolved in 100 µL of 10% dimethylsulfoxide (DMSO) in Phosphate buffered glucose saline (PBGS). 400 µL of PBGS was then added to produce extract solutions of 20.0 mg/mL (stock). Another extract concentration (10.0 mg/mL) was prepared from the first extract solution by appropriate dilution with PBGS. Aqueous extracts were dissolved directly in 500 µL PBGS. Extract solutions were prepared just before use. *In vitro* antitrypanosomal activity was performed in 96 well microtiter plates in triplicates. 20 µL of blood containing about 20-25 parasites per field was mixed with 5µL of extract solution of 20.0 mg/mL, 10.0 mg/mL and 2.0 mg/mL to produce effective test concentrations of 4 mg/mL, 2 mg/mL and 0.4 mg/mL, respectively. To ensure that the effect monitored was that of the extract alone, a set of control was included which contained the parasite suspended in 10% DMSO. For reference, tests were also performed with the same concentrations of Diminal®. After 5 min

incubation in closed Eppendorf tubes maintained at 37˚C, test mixtures were placed on separate microscope slides and covered with coverslips and the parasites were observed every 5 minutes for a total duration of sixty minutes. Cessation or drop in the motility of the parasites in extract-treated blood compared to that of parasite-loaded control blood without extract was taken as a measure of antitrypanosomal activity.

Statistical analysis

Results were recorded as mean ±SD and analyzed using SPSS version 25. One-way ANOVA was performed, followed by a multiple post hoc test where necessary. Values were considered significantly different at $p < 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the results of the qualitative phytochemical screening of various extracts of *Detarium microcarpum* leaves and stem bark. Polyphenols and flavonoids were detected in all extracts whereas terpenoid were only detected in the methanolic stem extract.

Table 1: Result of phytochemical screening of the various extracts of *Deterium microcarpum* leaves and stem bark

Phytochemical	ALE	ASE	HLE	HSE	MLE	MSE
Saponin	+	+	-	-	+	+
Tannins	-	+	-	-	+	+
Steroids	+	+	-	-	+	-
Glycosides	+	-	-	+	+	+
Alkaloids	+	+	+	+	+	+
Terpenoids	-	-	-	-	-	+
Polyphenols	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+

ALE = aqueous extract of leaves, ASE = aqueous stem bark extract, HLE = hexane leaves extract, HSE = hexane stem bark extract, MLE= methanol leaves extract, MSE= methanol stem bark extract.
+ indicates phytochemical detected, - Indicates phytochemical not detected.

Results are presented as mean ± standard deviation. Values with different superscripts are significantly different from each other across the row at $p<0.05$, GAE = Gallic acid equivalent. QE = Quercetin equivalent. Methanolic stem bark extract has the highest phenolic and flavonoid content.

Table 2. Shows the result for the determination of total phenolic and flavonoids content of various extracts of *detarium microcarpum* leaves and stem bark

Phytochemical	ALE	ASE	HLE	HSE	MLE	MSE
Phenolic (mg GAE/mg of extract)	110.62 ± 0.48^{a}	275.4 ± 0.16^{c}	143.62 ± 0.16^{b}	106.85 ± 2.04^{a}	205.7± 3.61^{d}	367.3± 15.84^{e}
Flavonoids (mg QE/mg of extracts)	8.70 ± 0.63^{b}	13.85 ± 0.41^{c}	109.44 ± 0.83^{e}	3.85 ± 0.41^{a}	19.57 ± 0.61^{d}	596.88 ± 0.13^{f}

ALE= Aqueous leave extract, ASE=Aqueous stem bark extract, HLE=Hexane leave extract, HSE=Hexane stem bark extract, MLE=Methanol leave extract, MSE=Methanol stem bark extract

Table 3. Shows the result for the *in vitro* antitrypanosomal activity of various extracts of *Detarium microcarpum* extracts (leaves and stem Bark) against *T.b brucei* and *T. congolense*

	Cessation time of parasite motility (in minutes)					
	T. b brucei			***T. congolense***		
Extract	2 mg/mL	10 mg/mL	20 mg/mL	2 mg/mL	10 mg/mL	20 mg/mL
ALE	NR	50**	40**	NR	40**	30**
ASE	NR	35**	25**	60*	30**	15**
HLE	25*	*35***	*25***	10*	25**	15**
HSE	20*	35**	*25***	10**	30**	20**
MLE	55**	20**	10**	55**	15**	5**
MSE	60*	35**	10**	55**	35**	25**
DPP	55**	*25***	*15***	60**	30**	20**

** indicates total cessation in motility (activity), * indicates a slight reduction in motility (activity).
DPP= Diminazene diaceturate + 555mg phenazone, ALE= Aqueous leaves extract, ASE=Aqueous stem bark extract, HLE=Hexane leaves extract, HSE=Hexane stem bark extract, MLE=Methanol leaves extract, MSE=Methanol stem bark extract, NR= no reduction in motility

From the table above, the extract ability to reduce trypanosome motility was seen to be both time concentration-dependent. When compared to the standard drug, methanolic leaves extract has the highest activity against *T. b. brucei* and *T.congolense*.

The figure below shows the DPPH Scavenging activity of various extracts of *Detarium microcarpum* leaves and stem bark.

There is no significant difference in the scavenging activities of the extracts when compared to ascorbic acid at $p<0.05$. This implies that the plant extracts possess the ability to scavenge free radicals.

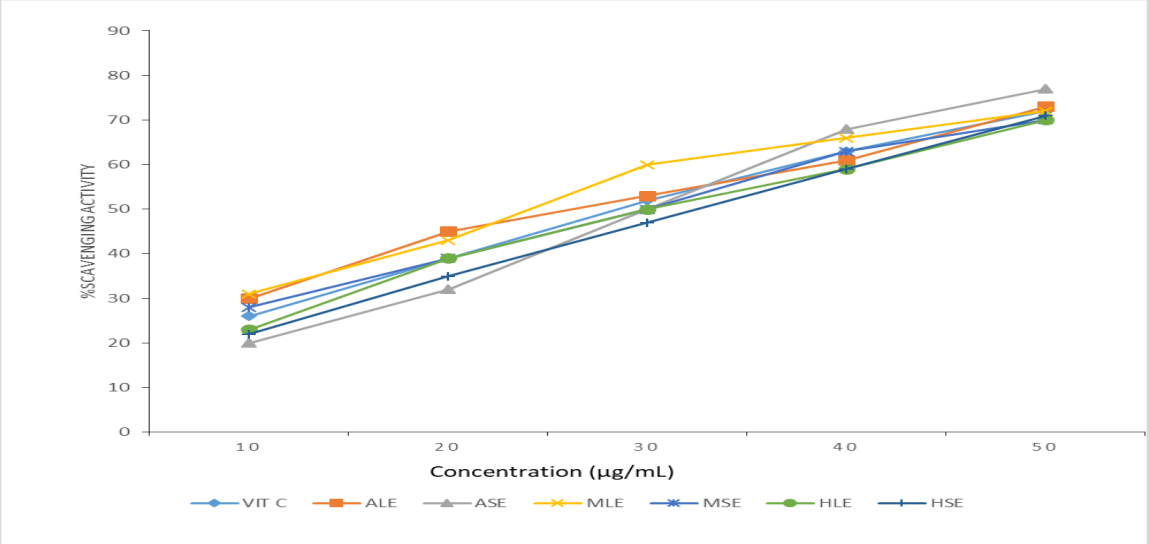

Figure 1. DPPH scavenging activity of various extracts of *Detarium microcarpum* leaves and stem bark compared with ascorbic acid

ALE= Aqueous leaves extract, ASE=Aqueous stem bark extract, HLE=Hexane leave extract, HSE=Hexane stem bark extract, MLE=Methanol leaves extract, MSE=Methanol stem bark extract.

The figure below shows the Hydrogen Peroxide scavenging activity of various extracts of *Detarium microcarpum* Leaves and Stem Bark compared with ascorbic acid. Extracts of aqueous stem bark, methanolic leaves and stem bark showed a significant difference ($p<0.05$) in H_2O_2 scavenging ability compared with ascorbic acid.

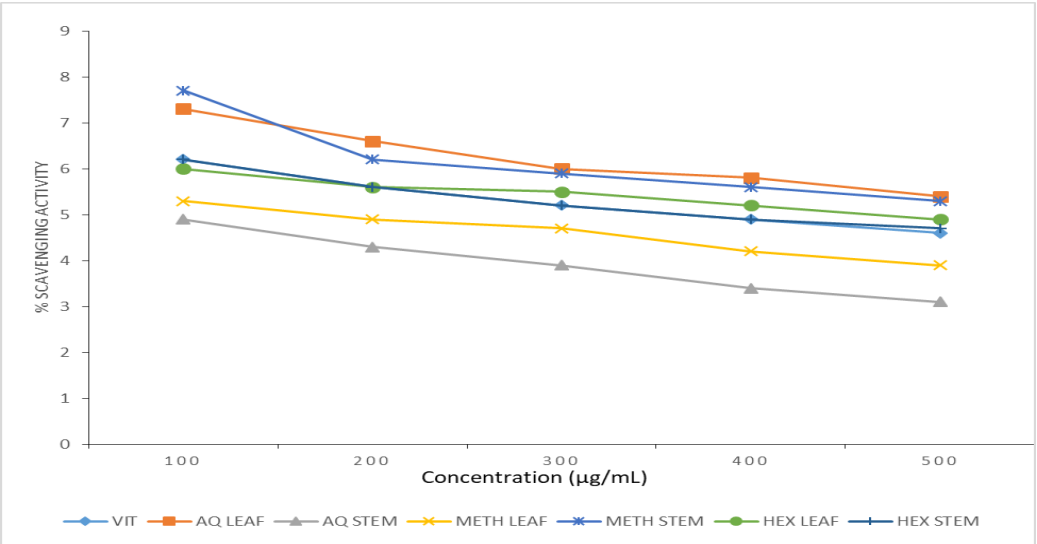

Figure 2. Hydrogen peroxide scavenging activity of various extracts of *Detarium microcarpum* leaves and stem bark compared with ascorbic acid

Vit = vitamin C, AQ LEAF= Aqueous extract of leaves, AQ Stem= Aqueous extract of stem bark, METH LEAF= methanolic extract of the leaves, METH STEM=methanolic extract of the stem bark, HEX LEAF=hexane extract of the leaves, HEX STEM= hexane extract of the stem bark.

Results are presented as mean ± standard deviation. Means not sharing the same letter are significantly different (Bonferroni multiple *post hoc* tests, $p<0.05$) in each column.

Table 4. Shows the result for the ic_{50} values of various extracts of *Detarium microcarpum* leaves and stem bark.

	IC_{50} Values (µg/mL)	
Standard/extract	DPPH	H_2O_2
Vitamin C	14.65±0.35[a]	1101.34±53.85[c]
Aqueous leave extract	27.75±0.07[c]	490.59±13.24[b]
Aqueous stem bark extract	30.55±0.35[d]	1227.04±77.19[c]
Hexane leave extract	31.85±0.35[de]	992.55±152.91[c]
Hexane stem bark extract	32.75±0.49[e]	665.34±63.96[b]
Methanol leaves extract	30.2±0.28[d]	109.61±13.38[a]
Methanol stem bark extract	24.95±0.07[b]	447.40±12.04[b]

Discussion

Medicinal plants since ancient time have been praised for their distinct therapeutic actions and these could be ascribed to the presence of some secondary metabolites such as glycosides, tanins, steroids, alkaloids, phenols etc. Some of these plants are important source of antioxidants by scavenging free radicals which are implicated in the pathogenesis of many diseases (Alhakmani *et al*., 2013).

The various extracts of *Detarium microcarpum* have been shown to contain phytochemicals such as tannins, saponins, flavonoids, alkaloids, glycosides, and phenolics which are bioactive compounds found in plants that confer medicinal properties to the plant, and this can be attributed to the fact that most of the phytochemicals screened are soluble in polar solvents. This result is in agreement with the previous report of Sani *et al.,* (2014,), David *et al.,* (2017). These phyto-compounds are important in the pharmaceutical industry for drug development and preparation of therapeutic agents. Steroids and terpenoids were not detected in this study, and this may be due to the fact that the study was done with crude extracts which may have complex composition and as such, some compounds may be masked.

The results from the *in vitro* antioxidant study showed that the various extracts of *Detarium microcarpum* exhibited antioxidant properties when compared to ascorbic acid. Gulcin *et al.,* (2004), Umamaheswari *et al.,* (2009) have shown a close relationship between total phenolics and flavonoids and antioxidant activity, since phenolic and flavonoid compounds serve as hydrogen donating agents. This link between these phytoconstituents of plants and their inhibitory activity against oxidants, therefore, necessitated the quantitative analysis of the various extracts of the leaf and stem bark of *Detarium microcarpum* for its polyphenol and flavonoid contents and from the study it was seen that polyphenols and flavonoids were present in various extracts of *Detarium microcarpum*. It was observed from the study that free radicals were scavenged by the extracts in a concentration dependent manner. The extracts' ability to donate hydrogen is assumed to be responsible for their ability to scavenge DPPH radicals. DPPH is a stable free radical that accepts an electron or hydrogen radical to form a diamagnetic molecule that is stable. The drop in DPPH radical absorbance at 517nm, was seen as a measure of their reduction ability. DPPH is a long-lived nitrogen radical that has no resemblance to the extremely reactive and transitory peroxyl radicals implicated in lipid peroxidation. Many antioxidants that respond swiftly with peroxyl radicals may react slowly with DPPH or may be inert (Ghaisas *et al.*, 2008). Although, there was no significant difference observed in the DPPH scavenging ability of the extracts compared with ascorbic acid, nevertheless, aqueous stem bark extract showed a significant difference in H_2O_2 scavenging ability when compared to ascorbic acid, with an IC_{50} value of 1227.04±77.19 µg/mL In the same vein, the methanolic extracts of the leaves and stem bark also showed a significant difference in H_2O_2 scavenging activities when compared to ascorbic acid with each having an IC_{50} value of 109.61±13.38 µg/mL and 447.40±12.04 µg/mL. Therefore, this implied that the plant extracts have good antioxidant capabilities.

From the *in vitro* antitrypanosomal activities of the various extracts, it can be seen that as the concentration of the various extracts increases, a reduction in motility of the parasites was observed as time progresses. The methanolic leave extract of *Detarium microcarpum* showed an efficient antitrypanosomal activity with complete cessation in motility at 5minutes against *T. congolense*, and at 10 minutes against T.*b brucei* at the highest concentration of the extract (20 mg/mL) when compared with the control. It took a long time for the complete cessation in the motility of the parasites at the concentration of 10 mg/mL of the extracts and no antitrypanosomal effect at the concentration of 2mg/mL for aqueous extracts of the leaves against the parasites. However, a slight reduction in motility at 60 minutes was observed in the aqueous extract of the stem bark against T. *congolense*. The drop in the motility of trypanosomes may serve as a measure of the antitrypanosomal potential of the plant extracts since parasite motility constitutes a relatively reliable indicator of the viability of most zoo-flagellate parasites (Peter *et al.*, 2012). The plant's ability to reduce trypanosome motility was seen to be both time and concentration dependent. In *in vitro* models, complete immobility of parasites does not suggest mortality of the parasites (*Tewabe et al*., 2014). A study conducted by Ibrahim *et al.,* 2020 on the methanolic

extract of *D. microcarpum* leaves against T. *congolense* showed that, the level of parasitemia was suppressed after 14days' treatment in a dose dependent fashion This result shows that *D. microcarpum* plant like many other plants in Nigeria has antitrypanosomal activity, though it is difficult to infer the mechanism by which these extracts exhibit their antitrypanosomal activity since the active ingredient(s) were not isolated. However, the plant may contain bioactive compound(s) that may be responsible for its trypanosuppressive activity. Sepulveda-Boaz and Cassels 1996 suggested that many natural products may be trypanocidal by virtue of their interference with the redox balance of the parasites either by acting on the respiratory chain or on the cellular defenses against oxidative stress. *In vitro* assays usually provide relatively reliable information for determining the extract's sensitivity on trypanosome isolates.

In conclusion, the results of this study showed that the various extracts of *Detarium microcarpum* possess possible inhibitory potential against free radicals in addition to its antitrypanosomal effect. Further research should be conducted to determine the *in vivo* antitrypanosomal effect of *Detarium microcarpum* leaves and stem bark extracts and to determine the active compound that may be responsible for the antitrypanosomal effect.

AUTHORS' CONTRIBUTIONS

The resaerch was conceptualized by author USN who also paricipated in the writing of the manuscript and in editing the final copy. Authors IS, SB, IA, AJK and AJ carried out the research and result analysis. Author ASK helped with the laboratory analysis while author JUE participated in the result analysis as well as preparation of the manuscript and submission of same for publication. All authors approved the final version for publication.

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CONFLICT OF INTEREST

The authors declare no conflict of interest

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