







An *In Vitro* Assessment of the Antioxidant Activity of *Detarium microcarpum* Guill. & Perr. Fabaceae

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[The author informations are in the declarations section. This article is published by ETFLIN in Sciences of Phytochemistry, Volume 3, Issue 2, 2024, Page 114-122. <https://doi.org/10.58920/sciphy0302267>]


Received: 24 July 2024

Revised: 20 October 2024

Accepted: 18 November 2024

Published: 27 November 2024

Editor: Samir Chtita

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Keywords: Free radicals, Lipid peroxidation, Oxidative stress, Phytochemical composition, Phytotherapy.

Abstract: Medicinal plants are regarded as important sources of exogenous antioxidants due to their phytoconstituents' free radical scavenging potential. The present study explores the phytoconstituents and antioxidant activity of *n*-hexane (HFDM) and chloroform (CFDM) fractions of *Detarium microcarpum* for potential use in the phytotherapy of oxidative stress-linked ailments. The phytoconstituents were qualitatively determined, while the antioxidant activity was determined by *in vitro* assays. Alkaloids, saponins, steroids, and flavonoids were detected in both fractions, while glycosides and terpenoids were absent. The HFDM (55.40 ± 2.66 AAE $\mu\text{g/mL}$) showed a significantly higher total antioxidant capacity than the CFDM (34.84 ± 1.22 AAE $\mu\text{g/mL}$, $p < 0.05$) at the tested concentration (100 $\mu\text{g/mL}$) while the CFDM (57.84 ± 2.16 AAE $\mu\text{g/mL}$) exhibited a significantly higher ferric reducing antioxidant power than the HFDM (46.11 ± 1.91 AAE $\mu\text{g/mL}$, $p < 0.05$) at the tested concentration (100 $\mu\text{g/mL}$). In the ferric thiocyanate assay, there was no significant ($p > 0.05$) difference between the HFDM ($65.59 \pm 2.32\%$) and CFDM ($77.42 \pm 2.95\%$). However, both fractions exhibited significantly higher percentage inhibition than ascorbic acid ($9.68 \pm 5.41\%$, $p < 0.05$). Moreover, the HFDM (0.21 ± 0.01 nmol/mL) exhibited a significantly lower MDA concentration than the CFDM (0.66 ± 0.06 nmol/mL) and AA (0.46 ± 0.02 nmol/mL). Additionally, ascorbic acid (0.46 ± 0.02 nmol/mL) showed a significantly lower MDA concentration than CFDM (0.66 ± 0.06 nmol/mL). The *n*-hexane and chloroform fractions of the plants showed promising antioxidant potential, which might be attributed to the identified phytochemicals that have potential applications in the phytotherapy of oxidative stress-linked diseases.

Introduction

Antioxidants are regarded as vital cellular components, widely recognized for their key role in maintaining redox balance and mitigating the damaging effects of free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), generated during normal cellular processes (1-6). The cell is inhabited by its inherent antioxidant enzymes, including glutathione, superoxide dismutase, and catalase, in addition to the exogenous antioxidant compounds such as ascorbic acid and tocopherol (3-5, 7). Antioxidants usually neutralize free radicals generated during normal cellular processes by donating electrons in some instances or by modulating pathways linked to the cellular processes (1, 3, 4, 8). However, in ailments

associated with increased free radical generation, including cancer and diabetes, a redox imbalance is established, leading to oxidative stress and depletion of the antioxidants (6, 9-12). Oxidative stress is a major component of many ailments, subsequently leading to inflammatory responses and promoting tissue damage, which might lead to fatal consequences (13-16). In diabetes, persistent hyperglycemia promotes oxidative stress *via* mitochondrial dysfunction, endoplasmic reticulum stress, chronic low-grade inflammation, and lipotoxicity, further exacerbating the condition and leading to microvascular and macrovascular complications (13, 17-19). A similar event occurs in cancer, including mitochondrial dysfunction and endoplasmic reticulum stress. However, there is an increased activity of ROS-

generating enzymes, including NADPH oxidase, cyclooxygenase (Cox), and xanthine oxidase in the cancer cells in addition to the Warburg effect, characterized by increased glucose metabolism even in the presence of oxygen (14, 16). Antioxidants play a vital role in health and diseases; however, their depletion in disease state might require augmentations from endogenous sources such as medicinal plants to mitigate oxidative stress.

Medicinal plants present a reservoir of plant-based drugs and natural products with varying pharmacological activities (20-28). Moreover, their abundance, affordability, and accessibility might provide an alternative to managing different ailments, including oxidative stress. In normal cellular conditions, ROS are generated as part of normal metabolic activities within the cell and subsequently neutralized to mitigate their damaging effects within the cell (29). However, the loss of cellular homeostasis due to the overproduction of ROS leads to harmful impacts on macromolecules, manifesting in ailments such as cancer and diabetes. Furthermore, this imbalance, coupled with the depletion of the inherent antioxidant system, further exacerbates these conditions, thus requiring an exogenous supply of antioxidants. In some reports, *in vitro* techniques were employed to determine the free radical scavenging activities of the plants, while other reports express the antioxidant effects *in silico*. In the *in vitro* techniques, including the ferric-reducing antioxidant assay and phosphomolybdate assay, different plant extracts and fractions are used in the presence of ferric and molybdate ions to explore the radical scavenging potential of the plants (24, 30-34). The *in silico* technique determines the modulative effects of compounds identified in the plants on the different proteins and enzymes associated with generating free radicals (28, 32, 33, 35). The therapeutic effects of medicinal plants are attributed to their phytochemical components, which they produce for plant growth and defence against predators and pathogens (21, 22, 26, 36-38). Phytochemicals, including saponins and flavonoids, are linked to different antioxidant activities *via* different mechanisms of action, such as direct free radical neutralization by donating electrons and modulating the activities of different cellular processes linked to free radical generation (39, 40).

D. microcarpum is a tree native to drier parts of Africa, including Nigeria, Sudan, and Senegal, growing up to 36 m with a thick and big crown, depending on the amount of rainfall in the area (41, 42). In folkloric medicine, different parts of the plant are prepared in different formulations, including decoction, infusion, and maceration, and they are employed in treating infections, fever, menstrual pains, and heart diseases (43). Different parts of the plant are documented to contain different phytochemicals with pharmacological

activities (44, 45), thus finding their way into different forms of phytotherapy. Additionally, some fatty acids, including linoleic, oleic, and palmitic acids, were previously identified in the plant and linked to potential antimicrobial activities of the plant (46, 47). The plant was previously linked to pharmacological activities, including antimicrobial, antiproliferative, antiplasmodial, analgesic, and anti-inflammatory (48-50, 45). Although the antioxidant activities of different parts of the plants were previously reported and attributed to their phytochemical components (51-53), there are still unexplored gaps in the quest to harness the full antioxidant potential of the plant. In the present study, the *n*-hexane and chloroform stem bark fractions of *D. microcarpum* were assessed for their phytochemical composition and free radical scavenging activity for potential application as antioxidants in oxidative stress-linked ailments.

Methodology

Materials

A sample of *D. microcarpum* was obtained from the Girei Local Government Area of Adamawa State, which was identified and authenticated by a Forest Technologist in the Forestry Technology Department of Adamawa State Polytechnic Yola. The sample was dried under shade, ground to powder, and stored till further use.

Extraction and Fractionation

The crude *D. microcarpum* extract was obtained by macerating the plant powder (one kilogram) in 75% (v/v) ethanol for 7 days with frequent agitation, followed by filtration with a Whatman No.1 filter paper to obtain the filtrate. The filtrate was dried over a rotary evaporator (Buchi Rotavapor R-200) under reduced pressure at 40°C to obtain 248 g of the crude extract. The crude extract was further subjected to fraction *via* solvent partitioning technique to get the *n*-hexane and chloroform fractions. Exactly 50 g of the crude extract was dissolved in 100 mL of distilled water and transferred to a separating funnel. An equal volume of *n*-hexane was added, shaken vigorously, and allowed to stand for separation. The *n*-hexane layer was collected, and the process was repeated until a clear *n*-hexane layer was observed. Furthermore, an equal volume of chloroform was added to the aqueous fraction and subjected to the same treatment as the *n*-hexane fraction. The *n*-hexane and chloroform layers were collected separately and dried *via* the same procedure for the crude extract to obtain the *n*-hexane (HFDM) and chloroform (CFDM) fractions, respectively.

Phytochemical Identification

The methods previously described by Evans (54) were adopted to detect the presence of alkaloids, saponins, steroids, glycosides, terpenoids, and flavonoids.

Alkaloids

Exactly 2 mL of 10% HCl was added to 2 mL of the extract, followed by the addition of 2 mL of Meyer's reagent. The formation of an orange precipitate indicated a positive result.

Saponins

Exactly 2 mL of distilled water was added to 2 mL of the extract, followed by 5 minutes of agitation. The appearance of a foam layer indicated a positive result.

Steroids

Exactly 10 mL of chloroform was added to 2 mL of the extract, followed by the addition of 10 mL of concentrated sulphuric acid by the side of the test tube. The formation of a reddish upper layer and yellow sulphuric acid layer with green fluorescence indicated a positive result.

Glycosides

Exactly 2 mL of acetic acid was added to 2 mL of the extract. The mixture was cooled in a cold water bath, and then 2 mL of concentrated H_2SO_4 was added. Color development from blue to bluish-green indicated the presence of glycosides.

Terpenoids

Exactly 2 mL of chloroform and 1 mL of concentrated sulphuric acid were carefully added to 2 mL of the extract to form a layer. A clear upper and lower layer with a reddish-brown interphase indicated a positive result.

Flavonoids

Exactly 2 mL of 10% sodium hydroxide was added to 2 mL of the extract. The formation of a yellow color, which turned colorless upon the addition of 2 mL of dilute hydrochloric acid, indicated a positive result.

Antioxidant Activity

Different methods were adopted to determine the *in vitro* free radical scavenging activity and anti-lipid peroxidation potential of the HFDM and CFDM.

Total Antioxidant Capacity

The TAC was determined by adopting the method previously described by Prieto et al. (55). Exactly 0.5 mL of distilled-water dissolved sample (300 μ g/mL) was mixed with 2 mL of phosphomolybdate reagent and capped, followed by 10 minutes of incubation at 95°C. The sample absorbance was read using a UV-Viz spectrophotometer (Model V1000) at 695 nm against a blank solution of phosphomolybdate reagent and distilled water subjected to the same treatment as the sample. Moreover, different concentrations (20 – 100 μ g/mL) of ascorbic acid (AA) were used to determine the AA calibration curve. The TAC was expressed as AA equivalent (AAE) in μ g/mL of triplicate determinations.

Ferric Reducing Antioxidant Power

The FRAP was determined as described previously by Oyaizu (56). Exactly 0.25 mL distilled-water dissolved sample (100 μ g/mL) was mixed with 0.625 mL of 1% potassium ferrocyanide and 0.625 mL of 0.2 M pH 6.6 phosphate buffer, followed by 20 minutes incubation at 95°C. Furthermore, 0.625 mL of 10% trichloro acetic acid (TCA) was added, followed by 20 minutes of centrifugation at 3000 rpm and collection of 1.8 mL of the upper layer, which was mixed with an equal volume of distilled water and 0.36 mL of 0.1% $FeCl_3$. The absorbance was read using a UV-Viz spectrophotometer (Model V1000) at 700 nm against a blank solution of reagents without the sample. The AA calibration curve was derived from different concentrations (20-100 μ g/mL) of AA, and the result was expressed as AAE μ g/mL of triplicate determinations.

Ferric Thiocyanate Assay

The anti-lipid peroxidation potential of the sample was determined by adopting the method of Kikuzaki et al. (57) via the FTC assay. Precisely 4 mL (1 mg/mL) and 4.1 mL of the sample and 2.25% (v/v) linoleic acid prepared in absolute ethanol were combined with 3.9 mL pH 7 phosphate buffer and 3.9 mL distilled water. This was followed by capping and 10 minutes of dark oven incubation at 40°C. Furthermore, 0.1 mL of the mixture was mixed with 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate, followed by the addition of 0.1 mL of 0.02 M ferrous chloride prepared in 3.5% HCl. The initial absorbance of the mixture was read using a UV-Viz spectrophotometer (Model V1000) at 532 nm three minutes after the addition of ferrous chloride. Moreover, a solution of the reagents without the sample was used as a control, while AA was standard. The percentage inhibition was determined according to Equation 1 and presented as a mean of triplicate determinations.

$$\%Inhibition = 100 - \left(\frac{A_t}{A_c} \times 100\% \right) \quad \text{Equation 1}$$

Where A_t = absorbance of sample and while A_c = Absorbance of control.

Thiobarbituric Acid Assay

The procedure previously described by Kikuzaki et al. (57) was adopted to further determine the anti-lipid peroxidation of the sample. One milliliter of the sample prepared in the FTC method was mixed with two milliliters of each of 20% TCA and 0.67% TBA, followed by capping and 10 minutes of incubation at 90 °C. The mixture was centrifuged at 3000 rpm for 20 minutes after cooling. The absorbance was read using a UV-Viz spectrophotometer (Model V1000) at 532 nm. Furthermore, a solution of the reagents without the sample is used as blank, while AA is standard. The

malonaldehyde (MDA) concentration was determined from the extinction coefficient 156 Mm⁻¹ cm⁻¹ using Equation 2.

$$MDA\ Level = \frac{A_t}{EC} \times V_s$$

Equation 2

Where At = absorbance of the sample, EC = extinction coefficient, and Vs = sample volume.

Statistical Analysis

The results obtained in the present study were expressed as the mean of ± standard error of triplicate determinations (±SEM). Moreover, the difference among the groups was analyzed with Statistical Package for the Social Sciences (SPSS) version 22 Software using an independent-sample T-test at p<0.05 significance level.

Result and Discussion

Phytochemical Identification

The phytochemical components detected in *D. microcarpum* are listed in Table 1. Both the HFDM and CFDM showed the presence of alkaloids, saponins, steroids, and flavonoids with the absence of glycosides and terpenoids. In our study, four classes of phytochemicals were identified in both fractions of *D. microcarpum*, including alkaloids, saponins, steroids, and flavonoids. The detection of similar phytochemicals in the HFDM and CFDM might be attributed to the polarity of the fractionating solvents, which isn't surprising, considering the polarity range of the solvents (58-60). Furthermore, the identified phytochemicals were previously detected. Dahiru et al. (45) reported similar results, detecting the presence of alkaloids, saponins, and flavonoids in *D. microcarpum* with the absence of steroids. Sanusi et al. (50) reported the presence of alkaloids, saponins, steroids, glycosides, and flavonoids, partially agreeing with our results. In another study, alkaloids, flavonoids, steroids, and saponins were reported, which agrees with our report (61). Additionally, the phytochemicals reported in our study were previously attributed to different pharmacological activities, including antioxidant activities.

Table 1. Phytochemical Components of *D. microcarpum*.

No.	Phytochemical	HFDM	CFDM
1	Alkaloids	+	+
2	Saponins	+	+
3	Steroids	+	+
4	Glycosides	-	-
5	Terpenoids	-	-
6	Flavonoids	+	+

Note: (+) = present, (-) = absent

Alkaloids exert antioxidant activity against oxidative

stress via modulation of pathways and enzymes linked to oxidative stress (62). Uthman et al. (63) reported concentration-dependent *in vitro* antioxidant activities of alkaloids and flavonoids from *Kalanchoe pinnata*, attributed to the identified compounds in the plant. In another report, alkaloid compounds isolated from *Litsea cubeba* demonstrated strong antioxidant activity *in vitro* which was linked to the functional groups present in the compounds (64). Lokesh et al. (65) reported the antioxidant activity of crude saponins from *Abutilon indicum* leaves with up to 96.16% free radical scavenging activity at 2.5 mg/mL concentration via 2,2-diphenyl-1-picrylhydrazyl scavenging assay. Crude saponins extracted from *Albuca bracteate* showed a significant free radical scavenging activity comparable to Rutin and Butylated hydroxytoluene via *in vitro* techniques (66). Xie et al. (67) reported the *in vitro* antioxidant activity of flavonoids extracted from *Cyclocarya paliurus* with a strong 2,2-diphenyl-1-picrylhydrazyl scavenging effect and IC₅₀ value of 0.146 mg/mL. In another report, flavonoids from *Moringa oleifera* leaf exhibited strong antioxidant and free radical scavenging potential via the FRAP and 2,2-diphenyl-1-picrylhydrazyl assays. Similarly, flavonoids with a quercetin basic structure showed the most active antioxidant activity via the FRAP assay with a significant anti-lipid peroxidation potential (68). In our study, these phytochemical components that might be linked to the free radical scavenging activity were identified, as reported in our result.

Antioxidant Activity

Total Antioxidant Capacity

The TAC of *D. microcarpum* is depicted in Figure 1, revealing the AA calibration curve and the TAC of the HFDM and CFDM of *D. microcarpum*. The HFDM (55.40 ± 2.66 AAE µg/mL) showed a significantly (p<0.05) higher TAC than the CFDM (34.84 ± 1.22 AAE µg/mL) at the tested concentration (100 µg/mL).

Ferric Reducing Antioxidant Power

Figure 2 depicts the FRAP of *D. microcarpum*, showing the AA calibration curve and the FRAP of the HFDM and CFDM. The CFDM (57.84 ± 2.16 AAE µg/mL) exhibited a significantly (p<0.05) higher FRAP than the HFDM (46.11 ± 1.91 AAE µg/mL) at the tested concentration, contrary to the result for the TAC.

Ferric Thiocyanate Assay

Figure 3 shows the anti-lipid peroxidation potential of *D. microcarpum* in the early stage of lipid peroxidation, revealing the percentage inhibitory effect of HFDM and CFDM. Although there was no significant (p>0.05) difference between the HFDM (65.59 ± 2.32%) and CFDM (77.42 ± 2.95%), both fractions exhibited significantly (p<0.05) higher percentage inhibition than AA (9.68 ± 5.41%).

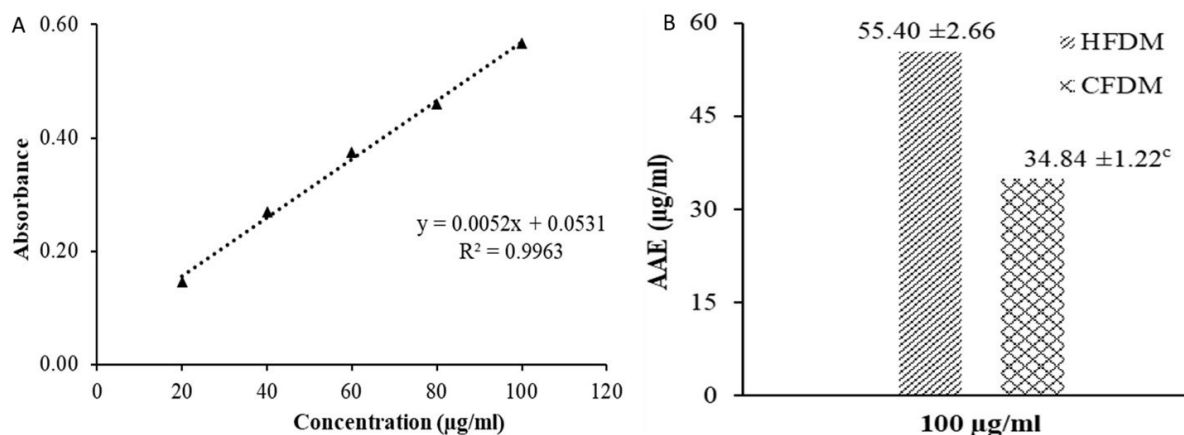


Figure 1. TAC of *D. microcarpum*. (A) AA calibration curve and (B) TAC of the HFDM and CFDM. (^c, $p < 0.05$) is significantly lower than HFDM.

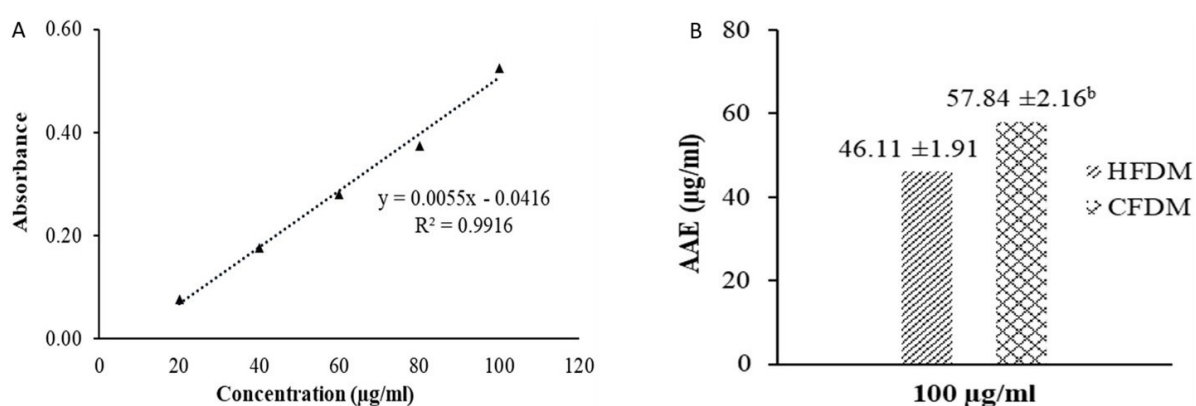


Figure 2. FRAP of *D. microcarpum*. (A) AA calibration curve and (B) FRAP of the HFDM and CFDM. (^b, $p < 0.00$) is significantly higher than HFDM.

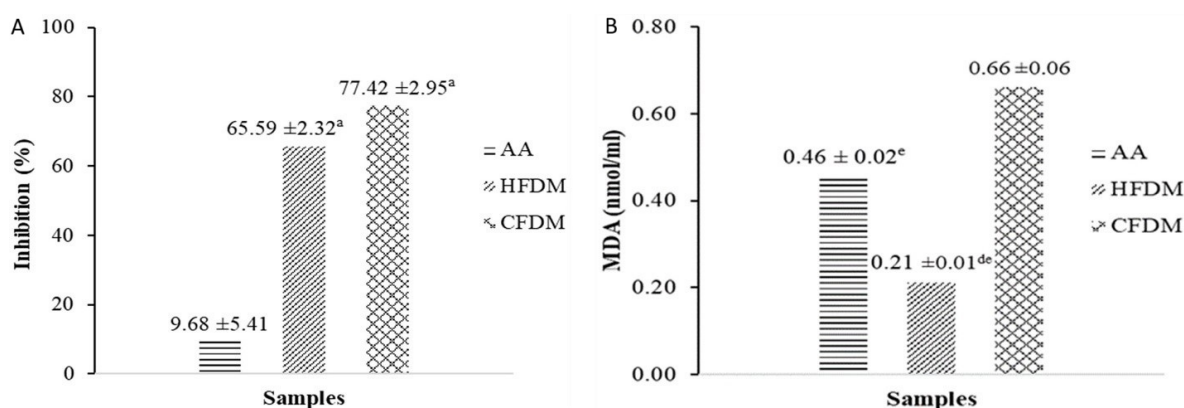


Figure 3. Anti-lipid peroxidation potential of *D. microcarpum*. (A) FTC assay and (B) TBA assay. (^a, $p < 0.05$) is significantly higher than AA while (^d and ^e, $p < 0.05$) are significantly lower than AA and CFDM, respectively.

Thiobarbituric Acid Assay

The effect of *D. microcarpum* on the late stage of lipid peroxidation (MDA formation) is shown in **Figure 3**, showing the MDA concentrations. The HFDM (0.21 ± 0.01 nmol/mL) exhibited a significantly lower MDA concentration than the CFDM (0.66 ± 0.06 nmol/mL)

and AA (0.46 ± 0.02 nmol/mL). Moreover, AA (0.46 ± 0.02 nmol/mL) showed a significantly lower MDA concentration than CFDM (0.66 ± 0.06 nmol/mL).

The present study explored the ability of *D. microcarpum* to scavenge free radicals and exert anti-

lipid peroxidation effects in vitro via different techniques. The antioxidant activity of plants might be influenced by their phytochemical composition, including the ones identified in our study (69). The radical scavenging activity of the HFDM was superior to that of the CFDM in the phosphomolybdate assay, which might be attributed to the presence of a higher number of electron-donating compounds in the HFDM. Moreover, the HFDM was initially fractionated from the crude extract based on polarity, which might lead to more of these compounds in the HFDM than the CFDM. In contrast, the CFDM showed superior activity in the FRAP assay, which might be due to the preference of the compounds present in the CFDM for the ferric ions over the Mo (VI) ions (70). The FRAP and phosphomolybdate assays are principled in reducing ferric to ferrous ions and Mo (VI) to Mo (V), respectively. Thus, both methods determine the radical scavenging potential of a test sample. In the FTC and TBA assay, the anti-lipid peroxidation showed the superiority of the CFDM in the early stage of lipid peroxidation; however, the HFDM exhibited superior activity in the late stage of lipid peroxidation. This might be related to the phytochemical components present in these fractions, which in turn influence the anti-lipid peroxidation potential of the fractions at different stages.

Conclusion

The present study explored the phytoconstituents and free radical scavenging potential of *D. microcarpum* for its potential application in phytotherapy of oxidative stress and related ailments. Conclusively, the *n*-hexane and chloroform fractions of the plants showed promising free radical scavenging potential, which might be attributed to the phytochemicals detected in the plant with potential application in the phytotherapy of diseases linked to oxidative stress. Moreover, both fractions might serve as an invaluable source of novel compounds with antioxidant activity. Thus, further studies to identify and isolate these compounds are warranted.

Abbreviations

HFDM = *n*-hexane Fraction of *D. microcarpum*; CFDM = Chloroform Fraction of *D. microcarpum*; MDA = Malonaldehyde; ROS = Reactive Oxygen Species; RNS = Reactive Nitrogen species; TAC = Total Antioxidant Capacity; FRAP = Ferric Reducing Antioxidant Power; FTC = Ferric Thiocyanate; TBA = Thiobarbituric Acid; SEM = Standard Error of Mean

Declarations

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Acknowledgment

The authors appreciate the contribution and institutional support of the Pharmaceutical Technology and Science Laboratory Technology Departments of Adamawa State Polytechnic, Yola.

Conflict of Interest

The authors declare no conflicting interest.

Data Availability

The unpublished data is available upon request to the corresponding author.

Ethics Statement

Not applicable.

Funding Information

Not applicable.

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How to cite: Dahiru, M.M., Oni, A.O., Danga, J., Alhaji, A.A., Jonah, F., Hauwa, A.Y., Muhammad, Z.. An In Vitro Assessment of the Antioxidant Activity of Detarium microcarpum Guill. & Perr. *Fabaceae. Sciences of Phytochemistry*. 2024; 3(2):114-122