Trop J Nat Prod Res, November 2022; 6(11):1844-1847
ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Short Communication

Comparative Antioxidant Activities of Leaf Extracts of *Detarium microcarpum Guill* & Perr (Fabaceae)

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ARTICLE INFO

Article history:
Received 21 May 2022
Revised 08 November 2022
Accepted 10 November 2022
Published online 01 December 2022

ABSTRACT

Detarium microcarpum is a plant that has been used widely to meet the nutritional needs of humans and also to treat various ailments in ethnomedicine. The present study is aimed at comparing the *in vitro* antioxidant activities of the leaves of *D. microcarpum* which have been extracted using solvents of varying polarities and also to determine the total phenolic and total flavonoid contents of the various extracts. The leaves of the plant were extracted successively using n-hexane, methanol, ethyl acetate, and water. The extracts' total phenolic and flavonoid contents were determined using a colorimetric assay. The antioxidant activity of the extracts was also determined using the 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), Nitric Oxide, and ferric reducing antioxidant power (FRAP) assay methods. All the extracts were found to have some phenolic and flavonoid content with the ethyl acetate extract having the highest total phenolic and total flavonoid contents ($p<0.05$). The different extracts demonstrated good antioxidant activities in the DPPH, nitric oxide, and FRAP assay methods.

Keywords: *Detarium microcarpum*, Antioxidant, DPPH, FRAP, Nitric Oxide.

Introduction

Detarium microcarpum Guill & Perr (family: Leguminosae) or "sweet dattock" is a medicinal plant that can be found growing wild in the tropical and Savannah regions of Africa.[1] It is an underutilized leguminous plant with both nutritional and medicinal properties in addition to industrial potential. The plant is widely distributed on the African continent and can be found in locations as diverse as Sudan, the Central African Republic, and various parts of Nigeria. Some of its local names are 'Taura' in Hausa, 'Ofor' in Igbo, and 'Ogbogbo' in the Yoruba language.[2] The plant has been shown to have tremendous economic and nutritional properties.[3] The seed contains proteins, minerals, vitamins, carbohydrates, fats, and soluble fiber and is widely used as a soup thickener.[4] Other uses of *D. microcarpum* include the use of the gum to improve the moisture retention of bread, thereby reducing the tendency to form crumbs[5], and as a binder in tablet manufacturing.[6] *D. microcarpum* is also used as an anti-feedant against termite[7], antidiabetic[8], anti-insect fumigant.[9, 10]. It is a good source of bioactive compounds as phytochemical screening of different parts of the plants has revealed the presence of saponins, alkaloids, tannins, and flavonoids. The plant has found use traditionally as a diuretic, anti-inflammatory,[11] and as anticancer agent.[12] It is used for treating malaria in Mali.[13]

Free radicals and reactive oxygen species (ROS) such as superoxide anion, and hydroxyl radicals, and non-radical species such as hydrogen peroxide and singlet oxygen are continually being produced in living cells due to metabolic activities.[14] These species possess significant toxicity and have been implicated in numerous degenerative conditions including arthritis, cancer, and diabetes mellitus.[15]

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Citation: David J, Agwom FM, Ojerinde SO, Ajima U, Alemika TE. Comparative Antioxidant Activities of Leaf Extracts of *Detarium microcarpum Guill* & Perr (Fabaceae). Trop J Nat Prod Res. 2022; 6(11):1844-1847. http://www.doi.org/10.26538/tjnpr/v6i11.16

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

The search for new therapeutic agents from natural sources that are effective at scavenging free radicals and cost-effective is therefore an exciting field that has the potential to contribute to the advancement of phytotherapy. The present investigation was aimed at evaluating the antioxidant property of various leaf extracts of *Detarium microcarpum*.

Materials and Methods

Plant Collection and Identification

The leaves of *D. microcarpum* were collected from Jos in North-central Nigeria in April 2018. The leaves were identified and authenticated at the Federal College of Forestry, Jos. A specimen with voucher number FHJ 234 was deposited in the herbarium of the Department of Pharmacognosy and Traditional Medicine of the University of Jos. The leaves were air-dried under a shade and subsequently pulverized.

Extraction

A 1-kilogram quantity of the powdered leaves was successively extracted using cold maceration with n-hexane, ethyl acetate, methanol, and water. The extracts obtained were collected and concentrated using a rotary evaporator at 40ºC. Concentrated extracts were then weighed and stored in a refrigerator before further studies.

Determination of Total Phenolic Content (TPC)

The Total Phenolic Content (TPC) of the extracts was evaluated using a colorimetric method utilizing the Folin-Ciocalteu reagent according to methods previously described with slight modification.[16] Samples containing polyphenols are reduced by the Folin-Ciocalteu reagent thereby producing a blue-colored complex. The phenolic concentration of *D. microcarpum* extracts was evaluated from a previously determined Gallic acid calibration curve. About 500 µl aliquots of 10, 20, 30, 40, 50, and 60 µg/ml of methanol Gallic acid solution were mixed with 2.5 ml Folin-Ciocalteu reagent (diluted ten-fold) and 2.5 ml (75 g/L) Sodium Carbonate. The tubes were vortexed for 10 seconds and allowed to stand for 2 hours at 25ºC. After incubation at 25ºC for 2 hours, absorbance was measured at 765 nm against reagent blanks. TPC was expressed as mg Gallic acid equivalent using the following equation based on the calibration curve;

$y = 0.0069x + 0.0673$, (correlation coefficient; $r^2 = 0.9947$)

Where x is the absorbance and y is the Gallic acid equivalent (mg/g). A similar procedure was adopted for the extract as described above for the preparation of the calibration curve. All determinations were carried out in triplicate. Total Phenolic Content (TPC) was expressed as milligrams of Gallic Acid Equivalent (GAE) per g of extract.

Determination of Total Flavonoid Content (TFC)

The Total flavonoid Content (TFC) of the *D. microcarpum* extracts was measured by employing an aluminum chloride colorimetric assay as reported.[16] An aliquot (1 ml) of extract (40 mg) or rutin standard solution with the following concentrations 10, 20, 40, 60, 80, and 100 µg/ml was measured into a 10 ml volumetric flask containing 4 ml of distilled water. A 300 µL of 5% $NaNO_2$ and 300 µL of 10% $AlCl_3$ were added to the flask. After 6 minutes, 2 ml of 1 M NaOH was added and the total volume was made-up to 10 ml by the addition of 2.4 ml H_2O. The solution was vortexed to mix the mixture thoroughly and the absorbance was measured at 510 nm against a reagent blank. The total flavonoid contents of the *D. microcarpum* extracts were expressed as mg rutin equivalent (RE)/g of extracts. All treatments were carried out in triplicate. The results were calculated using the standard calibration curve of rutin in methanol ($r^2 = 0.9957$).

Antioxidant screening: DPPH Method

The anti-oxidant activity (free radical scavenging activity) of the extracts on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined according to a previously described method.[17] Serial dilutions of the different extracts were prepared with methanol as solvent. Each prepared concentration (2 mL) was mixed with 4 mL of 50 µM DPPH solution in ethanol. The experiment was performed in triplicates and the mixture vortexed for 10 seconds to homogenize the mixture and then incubated for 30 minutes at room temperature in the dark. After incubation, the absorbance was then measured at 515 nm on a UV-Vis spectrophotometer. Lower absorbance of the resultant mixture indicates higher free radical scavenging activity. Ascorbic acid was used as the standard. A blank solution was prepared by mixing 2 mL of methanol with 4 mL of 50 µLM DPPH solutions. The difference in absorbance between the test and the control (DPPH in methanol) was calculated and expressed as % scavenging of DPPH radical. The capability to scavenge the DPPH radical is calculated as

$$\%\ inhibition = \left((Abs\ Control - Abs\ sample) \div Abs\ Control\right) \times 100$$

IC_{50}, defined as the concentration of the sample leading to a 50% reduction of the initial DPPH concentration, was also calculated from separated Linear regression of plots of the mean percentage of the antioxidant activity against the concentration of the test extract (µ/mL)

Antioxidant Screening: Nitric oxide scavenging assay

The method previously described[18] was employed in the study. Sample solutions were prepared from all the extracts of *D. microcarpum* leaves and dissolved in methanol. A 10 mg of each extract was dissolved in methanol and solutions of 50, 100, 150, and 200 mcg/ ml concentration were prepared. In this assay, 0.5 ml of Sodium nitroprusside (5 $mmolL^{-1}$) in phosphate-buffered saline pH 7.4, was mixed with different concentrations of the extract (50, 100, 150, and 200 mcg/ ml from a stock concentration of 100 mg/ ml methanol) and incubated at 25°C for °C for 180 minutes. The control was without the test compound, but an equivalent amount of methanol was taken.

After 3 h, 1.5 ml of Griess reagent (1% sulphanilamide, 2% phosphoric acid, and 0.1% *N*-1-naphthyl ethylene diamine dihydrochloride) was added and incubated for 30 minutes for color development. The absorbance of the chromophore formed during diazotization of the nitrate with sulphanilamide and subsequent coupling with *N*-1-naphthyl ethylene diamine dihydrochloride was measured at 546 nm and the percentage scavenging activity measured with reference to the standard.

The scavenging activity on the nitric oxide was expressed as inhibition percentage using the following equation:

$$\%\ Nitric\ oxide\ scavenging = \frac{absorbance\ of\ Control - Absorbance\ of\ test}{Absorbance\ of\ control} X\ 100$$

The tests were carried out in triplicate. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against extract concentration.

Antioxidant Screening: FRAP assay method

The FRAP reagent is made from 1:1:10 mixtures of three solutions: 20 mM $FeCl_3$, 10 mM TPTZ, and 0.3 M acetate buffer at pH 3.6 to give a light blue-purple color solution. The FRAP reagent prepared was used within 24 hours. To prepare the extracts, three portions of 0.05 g of the sample were added to 3 separate vials placed in a parallel reactor of 60^oC for 90 minutes, the extinction coefficient (e) for Fe (II) is obtained from the standard curve using $FeSO_4$ solution. $FeSO_4$ solutions are prepared as a specific concentration by dissolving 0.112g $FeSO_4.7H_2O$ in 200 ml degassed water. For the calibration curve 2500 µL FRAP reagent, 275 µL distilled water, and 60 µL of the $FeSO_4$ solutions were added to a 3 ml cuvette, or in a 500:55:12 ratio respectively. After extracts have been in the parallel reactor for 90 minutes, FRAP reagent 330 µL water and 70 µL of the extract are then left for 30 minutes before absorbance is measured. The generation of the indigo Fe^{2+}-TPTZ complex in the FRAP reagent from the clear Fe^{3+}-TPTZ complex can be easily observed in this state.

The spectrometer was calibrated using a blank cuvette of 30 ml FRAP reagent and 330 µL of distilled water. A diluted blank cuvette was prepared with 3.060 mL distilled water and 330 µL FRAP reagent or in other words, with a 100:11 mix of FRAP reagent and distilled water. It is used for an extract that required a 0.1 dilution factor to be read by the spectrometer. After 30 minutes the 20 mL vials of FRAP reagent and extract were obtained. The absorbance of solutions containing extract and FRAP solution were measured at 595 nm. The absorbance and Beer-Lambert law are utilized in the determination of the extinction coefficient. The proportion of Fe^{3+} reduced to Fe^{2+} concentration was then calculated.[19]

Statistical analysis

The results obtained are expressed as mean ± SEM. One-way analysis of variance (ANOVA) followed by Dunnets' posthoc test was used to determine if they were statistically significant differences between treated groups. P-values < 0.05 were considered significant. Statistical Package for Social Scientists (SPSS) Version 22.0 software was used for all statistical analyses.

Results and Discussion

Earlier research work had shown that *D. microcarpum* contains several phytochemicals, which have been found useful in mitigating oxidative damage linked to certain disease conditions. Some of the phytochemicals that have been reported in the leaf extract include saponins, tannins, flavonoids, cardiac glycosides, steroids, and terpene.[20-22] Phenols and flavonoids are secondary metabolites in plants and have been proven to possess a wide range of therapeutic activities such as anti-oxidant, anti-inflammatory, and anti-cancer properties.[23, 24] Phenolic compounds have also been identified as the major phytochemical responsible for anti-inflammatory and antioxidant activities.[25] Flavonoids on the other hand can similarly be considered phenolic in nature, and their antioxidant activity is greatly influenced by the substitution which affects the stability of the phenoxyl radical.[26] The interest in phenolics as potent antioxidants and free radical scavengers is growing. The ability of phenolics to act as reducing agents, singlet oxygen quenchers, hydrogen donors, lipid peroxidation stabilizers as well as inhibition of oxidizing enzymes have been identified as the plausible mechanisms for the antioxidant activity of the phenolic compounds.[27] In this study, since the ethyl acetate extract had the highest values of phenolic and flavonoid content, it is also expected to also demonstrate the best antioxidant activity. From Table 1, the ethyl acetate extract had the highest values of both flavonoid and phenols respectively.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 1: Total flavonoid and phenolic content of the various *D. microcarpum* leaf extracts

Extract	Total Flavonoid (mgRE/g extract)	Total Phenol (mgGAE/g extract)
n hexane	1.30 ± 0.05[a]	9.49 ± 0.06[a]
Ethyl acetate	268.85 ± 0.14[b]	11.35 ± 0.13[b]
Methanol	164.73 ± 0.10[a]	9.10 ± 1.83[a]
Aqueous	23.47 ± 0.89[a]	7.23 ± 0.09[a]

Means tagged with different letter alphabet in the same column are significant at ($p<0.05$); Values are mean±SEM, n=3

Table 2: Antioxidant activity of the various extracts of *D. microcarpum*

Extract	DPPH IC_{50} (µg/ml)	Nitric Oxide IC_{50} (µg/ml)	FRAP EC_{50}(µg/ml)
Aqueous	7.61±0.26	208.93±0.63	4.73±0.36*
Methanol	2.74±0.18*	103.50±0.68*	10.2±0.10*
Ethyl acetate	2.93±0.25*	37.42±0.95*	22±2.00
n-hexane	5.50±0.25*	113.38±0.95*	29±.4.00
RUTIN (STD)	ND	40.01±0.57	ND
Vit-C (STD)	0.50±0.07	ND	68.53±0.53

Values are expressed as mean ± S.E.M., n = 3.
*Significantly different from control: P< 0.05 ND = Not Determined

This result is similar to previous research where the acetone extract of *D. microcarpum* fruit was also found to contain more phenolic content than the methanol extract[28] showing that solvents of intermediate polarity are excellent at extracting these bioactive constituents. The value of total phenolic content (TPC) obtained for the various extracts in this study ranged between 7.23-11.35 mg Gallic acid equivalent/g and is significantly lower than that obtained for the root extract of *D. tripetala* in another study.[29] In the present study, three different antioxidant models (DPPH, Nitric Oxide, and FRAP) involving different mechanisms were used to evaluate the antioxidant activity of the n-hexane, ethyl acetate, methanol, and aqueous leaf extracts of *D. microcarpum.* DPPH is a nitrogen-centered free radical which is stable at room temperature and the results for the antioxidant activity by this model showed that the methanol extract had the highest antioxidant activity with IC_{50} (µg/ml) of 2.74±0.18 as compared to ascorbic acid (0.50±0.07). Furthermore, antioxidant evaluation using the Nitric Oxide model revealed that the ethyl acetate extract had the strongest activity with an IC_{50} value of 37.42±0.95 compared to 40.01±0.57 for the standard rutin. On the other hand, the FRAP assay revealed the aqueous extract as having the highest antioxidant activity with IC_{50} 4.73±0.36 µg/ml as compared to 68.53±5.43 for ascorbic acid. These differences are likely due to the different mechanisms of antioxidant activity employed by the three different assays. The result of the DPPH free radical scavenging activity obtained in this study was found to be similar to a previous study where the ethyl acetate and n-butanol extracts of the plant were demonstrated to possess greater DDPH free radical scavenging activity as compared to the methanol and hexane fractions of *D. microcarpum*.[17] The antioxidant activity by the DPPH method obtained in this study is also in close agreement with that obtained in a study on the stem bark of *Pycnanthus angolensis* where the ethyl acetate and methanol fractions were found to possess the strongest antioxidant activity.[30] Some possible phenolic compounds that have been identified as being responsible for the antioxidant activity of the *D. microcarpum* leaves include Gallic acid, quercetin-3,7-*O*-dirhamnoside, myricetin-3-*O*-rhamnoside, quercetin-3-*O*-rhamnoside, quercetin-3-*O*-glucoside, and kaemferol-3-*O*-Rhamnoside.[17]

Conclusion

This study has highlighted the antioxidant properties of the leaves of *D. microcarpum* extracted using a variety of solvents ranging from non-polar to polar. The concentrations of the phenolic and flavonoid compounds which may be responsible for the antioxidant activity of the plant extract were highest in the ethyl acetate and methanol extracts and the antioxidant activities of the leaf extracts also positively correlated with this.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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