Trop J Phytochem Pharm Sci, September 2024; 3(6): 345 - 349 **ISSN 2955-1226 (Print)**
ISSN 2955-1234 (Electronic)

Tropical Journal of Phytochemistry & Pharmaceutical Sciences

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

Original Research Article

Antioxidant Properties of Ethanol Crude Extract, Partitioned and Chromatographic Fractions of *Acacia ataxacantha* DC (Fabaceae) stem bark

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ABSTRACT

Acacia ataxacantha is used in the management of several diseases in many parts of Nigeria. The study was carried out to evaluate the antioxidant potential of ethanol crude extract, partitioned and chromatographic fractions of the stem bark of *Acacia ataxacantha.* The dried powdered stem bark was extracted with 70% ethanol and the extract was partitioned with ethyl acetate (EA) and 1-butanol (BT) in succession. The EA fraction was subjected to flash column chromatography while the BT fraction was further fractionated by gel filtration using sephadex LH_{20}. The eluates from the EA fraction eluted with dichloromethane /ethyl acetate (95:5) produced a single spot in iodine tank and were pooled together to give a white solid (EAF). The eluates from the BT fraction which produced one spot in iodine tank were pulled together and coded BTF. The crude extract, EA, BT, EAF and BTF were screened for antioxidant properties using 2, 2- diphenyl-1-picrylhydrazyl (DPPH), 2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) assay procedures with ascorbic acid (AA) used as the standard. The crude extract produced the greatest inhibition in the ABTS assay with IC_{50} of 0.46±0.03 while the EAF produced the least activity in the FRAP assay with IC_{50} of 37.45±0.06. The crude extract and BF produced the best antioxidant properties in all the assay methods employed hence, the plant has the potential to be developed into an antioxidant compounds.

Keywords: *Acacia ataxacantha*, antioxidant, 2, 2- diphenyl-1-picrylhydrazyl (DPPH), 2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS), Ferric reducing antioxidant power (FRAP), Column chromatography

Received 26 July 2024
Revised 12 September 2024
Accepted 12 September 2024
Published online 01 October 2024

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Introduction

Oxidative stress is implicated in several disease conditions viz; inflammation, cancer, diabetes and neurodegenerative disorders such as alzheimier and so on.[1] Medicinal plants are a potential source of natural antioxidants and are more suitable in the management of such chronic diseases than synthetic ones which have diverse side effects.[2] Plant secondary metabolites such as flavonoids and others are responsible for the properties possessed by medicinal plants. Some organic and inorganic compounds such as coumarins, phenolic acids and antioxidants micronutrients; Cu, Mg, Zn also contribute to the efficacy of most medicinal plants.[3]

The genus acacia, of the family, Fabaceae comprised of 1,200 species;[4] which are distributed in the tropical countries of West and other parts of Africa sub-continent; especially Benin, Angola, Botswana, Cameroon, Burkina Faso, Central African Republic, Guinea, Chad, Ivory Coast, Liberia, Kenya, Mali, Namibia, Mozambique, Nigeria, Niger, Senegal, South Africa, Sierra Leone, Swaziland, Togo, Tanzania, Zimbabwe and Zambia. [5]

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Citation: Baba H, Tarfa F, Lawal BA, Akpanika GA, Ahmadu AA. Antioxidant Properties of Ethanol Crude Extract, Partitioned and Chromatographic Fractions of *Acacia ataxacantha* DC (Fabaceae) stem bark. Trop J Phytochem Pharm. Sci. 2023; 2(6): 345 - 349 http://www.doi.org/10.26538/tjpps/v3i6.4

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

The different species are reported to be used in ethnomedicine for the treatment of varied ailments such as dysentery, cough, pain and inflammation, pneumonia, diabetes, acquired immunodeficiency syndrome (AIDS) and human immunodeficiency virus (HIV) opportunistic infections. [5, 6]

Phytochemical screening of the bark and root extracts (chloroform, dichloromethane, ethanol, ethyl acetate, hexane, hydroalcoholic, petroleum ether and methanol) of *A.ataxacantha* have been reported to contain anthracene derivatives, alkaloid, carbohydrates, coumarins, flavonoids, lignan, naphtoquinone, polyphenols, reducing sugars, saponins, steroids, tannins, terpenoids and triterpenoids.[7-12]

Acacia ataxacantha DC., has been reported to exhibit antimicrobial activities, hypoglycaemic effect and antioxidant properties.[5] Previous phytochemical works on this specie have reported the isolation of triterpenes and steroids, [15, 16] while a ferulic acid ester was isolated from the chloroform extract of the stem bark.[17] Also reported is a new chromone derivative referred to as acthaside.[18] Amoussa *et al.*[18] isolated betulinic acid-3-trans-caffeate, betulinic acid, lupeol. Aba *et al.*[10] isolated α-amyrenol (3*β*)-Urs-12-en-3-ol from root bark extracts of *A. ataxacantha.* We have previously reported the protein kinase inhibitory properties of extracts, fractions and isolated compounds from some acacia species.[19-21] The antioxidant potentials of extracts (hexane, dichloromethane, ethyl acetate, methanol, ethanol/water mixture) but not column fractions of the stem bark of *A. ataxacantha* using DPPH and FRAP assays including total phenolic content, flavonoids and flavanols have been reported.[9]

In this present study, as part of the continuous work on the plant *A.ataxacantha*, the antioxidant properties using DPPH, ABTS and FRAP assays, of the ethanol crude extract, ethyl acetate and n-butanol soluble fractions as well as a chromatographic fractions of the stem bark of *Acacia ataxacantha* is reported herein.

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ISSN 2955-1226 (Print)
ISSN 2955-1234 (Electronic)

Materials and Methods

Plant material

The plant material (*A. ataxacantha* stem bark) was collected in Jimba-Oja community, Ifelodun Local Government Area of Kwara State, Nigeria, in November, 2021. The plant was identified by Mr. Bolu Ajayi, a plant taxonomist at the Department of Plant Biology, University of Ilorin, Nigeria. The stem bark was air-dried and pulverized into coarse powder using an electric milling machine.

Extraction and Fractionation

The pulverized plant material (500 g) was macerated with 70% ethanol at 25 ^{o}c 7 days. The combined aqueous-ethanol extracts was concentrated *in vacuo* using a rotary evaporator; the temperature of the water-bath was set at 40°C, to give a dried extract of solid mass (11 g). The concentrated extract (10 g) was dissolved in distilled water (100 mL) and successfully partitioned with ethyl acetate (3 x 100 mL) and 1-butanol (3 x 100 mL). The ethyl acetate (EA) and 1-butanol (BT) fractions were dried and kept in a desiccator. The crude extract, EA and BT fractions were subjected to antioxidant procedures using DPPH, ABTS and FRAP assays. The EA solvent fraction was loaded on a flash column, with column silica gel G 200 – 400 mesh and eluted with dichloromethane and dichloromethane-ethyl acetate mixtures as follows; dichloromethane (100%), 99:1, 98:2, 97:3, 96:4, 95:5, 94:6, 93:7, 92:8, 91:9, 90:10, 80:20, 70:30, 50:50, 30:70, 10:90 and 100% (ethyl acetate). The column fractions were monitored with precoated thin layer chromatography (TLC) plates and spots detected in iodine tank. The column eluates with similar TLC profile were combined. The eluates collected at solvent ratio (95:5) gave a single spot on the TLC but could not be established as a pure compound. It was coded EAF and set aside for antioxidant assay. The BT solvent fraction was further purified by gel filtration using sephadex LH_{20} and methanol as mobile phase and the eluates were equally monitored with TLC method and spots detected in iodine tank. A series of fractions produced a clean spot on the TLC but could not be established as a single compound and they were combined and coded BTF which was set aside for antioxidant assay.

Antioxidant activity Determination

The DPPH assay was done according to the method of Brand-Williams *et al.*[22] with some modifications. 2, 2- diphenyl-1-picrylhydrazyl (DPPH) 8mg was dissolved in MeOH (100 mL) for a solution concentration of 80 μg/mL. To determine the scavenging activity, 100 μL DPPH reagent was mixed with 100 μL of sample in a 96-well microplate and was incubated at room temperature for 30 min. After incubation, the absorbance was measured at 514 nm using a microplate reader, and 100% methanol was used as a control. The DPPH scavenging effect was estimated using the following formula:[34]

$$\%\,DPPH\ scavenging\ = \frac{Ablank - Asample}{(Ablank)})\,x100. \qquad (1)$$

Where A_{blank} represents the absorbance of the control reaction (containing all reagents except the test sample) and A_{sample} represents the absorbance of the test sample/standard.

For ABTS assay, the procedure followed the method of Arnao *et al.*[23] with some modifications. 7mM 2, 2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) and 2.45mM potassium persulfate were mixed and dissolved in double distilled water. The solution was then diluted with distilled water in a 1:9 v/v ratio. A 190 µl volume of reagent was pipetted into a microtiter well with succeeding addition of 10 µl of sample/standard (ascorbic acid). Absorbance was measured at λ=735 nm. A reagent blank reading was taken and after addition of sample, the absorbance reading was taken after 6 minutes of initial mixing. The antioxidant activity was estimated using the following formula.

$$\%\,ABTS\ scavenging\ = \frac{Ablank - Asample}{(Ablank)}\,x\,100 \qquad (2)$$

For the FRAP assay, the solutions were prepared as described previously.[24,25] The FRAP working solution was prepared by mixing 10-volumes of acetate buffer (300 mM, pH 3.6) with 1- volume of 2,4,6-tripyridyl-S-triazine (TPTZ) (40mM dissolved with 40mM HCl) and 1-volume of ferric chloride (20 mM in water). Microplate FRAP assay was performed according to previous reports with minor modifications.[26, 27] Sample solutions (20 µl) were added directly to the 96-well microplate followed by 280 µl of working FRAP solution. The mixtures were shaken, incubated at 37°C in the dark for 30 minutes and then absorbance was taken at 593nm using a microplate reader. The values were estimated in µM ferrous (Fe II) equivalent.

Statistical analysis

Data were presented as mean ± SEM. The graphical representation of the data was performed using Microsoft excel 2016 (Microsoft USA). The difference was considered statistically significant when the $p < 0.05$.

Results and Discussion

The results of the antioxidant assay for the crude extract, EA, BT, EAF, BTF and ascorbic acid (standard) using DPPH, ABTS and FRAP assay methods are shown in figures 1, 2 and 3 respectively. The IC_{50} (i.e. the concentration that gives 50% inhibition) was calculated using regression equation and summarized in table 1.

EA: ethyl acetate fraction, BT: 1- butanol fraction, EAF: ethyl acetate column fraction, BTF: 1-butanol column fraction. Values are mean ± S.D (n =3)

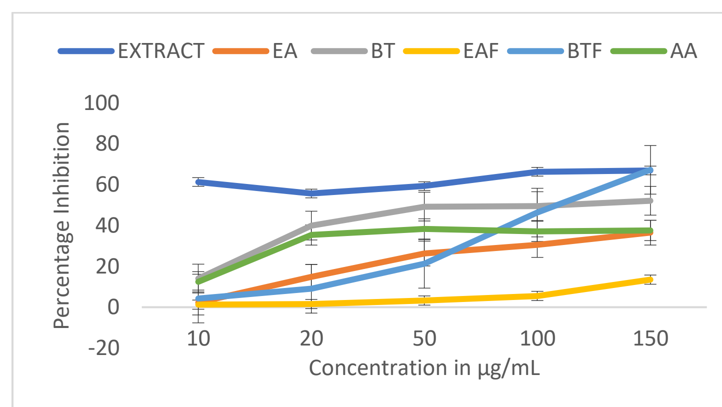

Figure 1: Per cent (%) DPPH scavenging effect of the crude extract, ethyl acetate (EA), 1-butanol (BT), ethyl acetate column fraction (EAF), 1-butanol column fraction (BTF) fractions and ascorbic acid (AA). Values are given as mean ± S.E.M.

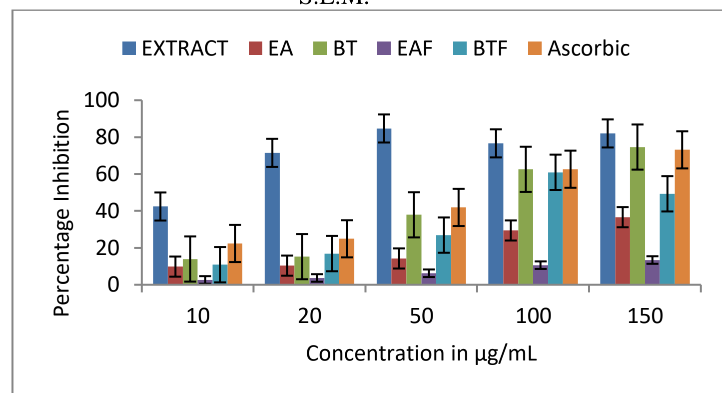

Figure 2: Per cent (%) ABTS activity of the crude extract, ethyl acetate (EA), 1- butanol (BT), ethyl acetate column fraction (EAF), 1- butanol column fraction (BTF) and ascorbic acid. Values are given as mean ± S.E.M.

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ISSN 2955-1226 (Print)
ISSN 2955-1234 (Electronic)

DPPH assay

DPPH is a stable free radical that accepts an electron or hydrogen to become a stable diamagnetic molecule. When antioxidants interact with DPPH, they transfer electron or hydrogen atom to DPPH and thus neutralizing its free radical character and convert it to 1 - 1 diphenyl- 2 - picryl hydrazine and the degree of discoloration indicates the scavenging activity of the drug.[28] The reduction of DPPH radical by antioxidants is determined by the decrease in absorbance at 517 nm. The decrease in absorbance of DPPH radical caused by antioxidants is due to the reaction between antioxidant molecules and radical progress which results in the scavenging of the radical by hydrogen donation. [29] It is visually noticeable as a change in colour from purple to yellow. Hence, DPPH is usually used as a substance to evaluate the antioxidant potential of medicinal plants.[30] The effectiveness of antioxidant activity is revealed by estimating the IC_{50}; which is the concentration of the extract that produced 50% reduction of the DPPH radical. The lower the IC_{50}, the higher the inhibitory activity. The crude ethanol extract showed the highest inhibitory activity with IC_{50} of 2.44±0.04 µg/mL followed by BT; 4.05 ± 0.06, while EAF with IC_{50} of 18.80 ± 0.12 has the least inhibitory activity. It is evidenced from the results that the crude extract and even the BT fraction are more active in reducing the DPPH radical than the standard drug; ascorbic acid. Obviously, the more polar fractions (BT and BTF) were found to demonstrate better inhibitory activity than the less polar fractions (EA and EAF). This corroborate an earlier result obtained ~~on~~ from the same plant.[9] The DPPH scavenging activity of the solvent and column fractions were found to be dose dependent except for the crude extract that was found not to increase with increase in dose. There could be several factors responsible for the anomaly, one of such factors is saturation effect. That is to say, the extract reached a saturation point beyond which increasing the dose does not lead to a proportional increase in activity.

ABTS assay

According to the ABTS method, pre-formed dark green stable free radical 2,2'-azino-bis(3ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation (ABTS•+) is reduced by a compound.[31] From figure 2, the extract produced the highest percentage inhibition all through the concentration range but it is non dose dependent. The highest inhibition by the extract was found to be at 50 µg/mL. This could mean that the extract reached the point of saturation at 50 µg/mL. The BF and AA exhibited a dose dependent percentage inhibition as the EAF was found to produce the least inhibition. There is similarity between the results of DPPH assay and that of ABTS; as the activity of the extract is found to be dominant in the more polar butanol fractions. The ethyl acetate fraction produced the least inhibition of the radical activity in both cases.

FRAP assay method

The reducing capacity of the extracts is another significant indicator of antioxidant activity. The presence of antioxidants in the sample would result in the reduction of Fe^{3+} to Fe^{2+} by donating an electron which is an important mechanism of phenolic antioxidant action[32] In this assay, the compounds with reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanides (Fe^{2+}), which then react with ferric chloride to form ferric ferrous complex that is greenish in colour.[33] The FRAP assay result illustrated in figure 3 was extrapolated and estimated as equivalent of ferrous sulphate using a straight line graph (figure 4)

$$y = 0.0002x + 0.0411, R^2 = 0.9564 \quad (3)$$

From the result illustrated on the charts, the crude extract was still found to be more active than the different partitioned and column fractions as witnessed under the DPPH and ABTS assay methods. The slight difference however, from the rest of the assay methods is the fact that the EA was seen to exhibit a considerable activity under this assay method. Overall, all the fractions including the standard substance used appear not to exhibit serious inhibitory activity under the FRAP assay method. The pattern of activity seen in these different assay methods is not surprising as their mechanisms of activity differ.[22]

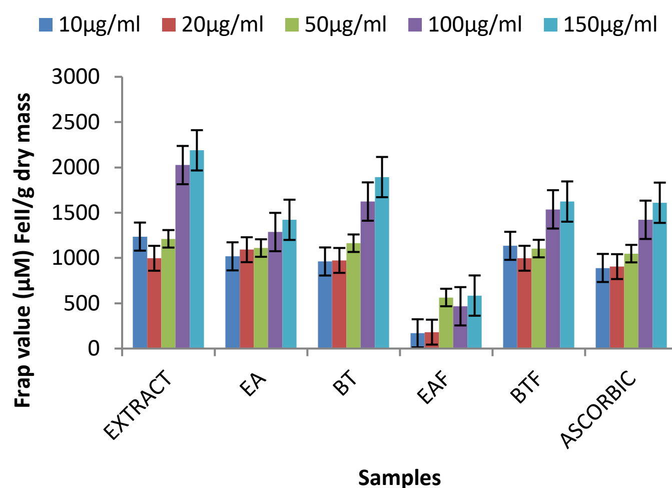

Figure 3: FRAP activity of the crude extract, ethyl acetate (EA), 1-butanol (BT), ethyl acetate column fraction (EAF) 1-butanol column fraction (BTF) and ascorbic acid. Values are given as mean ± S.E.M.

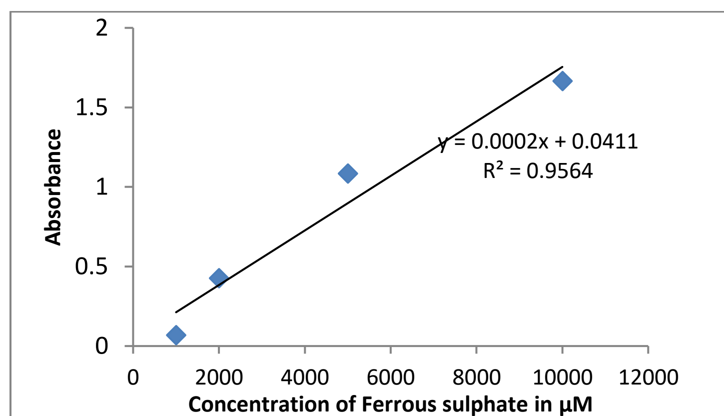

Figure 4: Graph of Absorbance against Concentration of Ferrous Sulphate

Table 1: $IC_{50\ values}$ (µg/mL) of the ethanol crude extract, fractions and ascorbic acid

	DPPH	ABTS	FRAP
Extract	2.44 ± 0.04	0.46 ± 0.03	6.77 ± 0.04
EA	6.32 ± 0.04	7.11 ± 0.03	33.54± 0.03
BT	4.05 ± 0.06	3.36 ± 0.06	14.63 ± 0.04
EAF	18.80 ± 0.12	17.97 ± 0.06	37.45 ± 0.06
BTF	4.25 ± 0.06	4.41± 0.08	12.02 ± 0.11

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 ISSN 2955-1226 (Print)
ISSN 2955-1234 (Electronic)

Ascorbic acid	6.43 ± 0.06	3.54 ± 0.06	18.69 ± 0.02

EA: ethyl acetate partitioned fraction, BT: 1-butanol partitioned fraction, EAF: ethyl acetate column fraction, BTF: 1-butanol column fraction. Values are given as mean ± S.E.M (n = 3).

Conclusion

The activity of the extract and fractions of the stem bark of *A.ataxacantha,* especially the 1-butanol fraction is quite impressive. This may explain the various uses this plant is involved in ethno-medicine. Further purification on the 1-butanol fraction work is currently ongoing in our laboratory in a bid to isolate the active compounds which could be responsible for this interesting activity.

Conflict of Interest

The authors declare that there was no conflict of interest.

Authors' Declaration

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

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