NOVEL ANTIOXIDANT ACTIVITY OF *CYATHULA PROSTRATA (L.) BLUME*)

Olawale H. Oladimeji,[a,]* Cyril O. Usifoh[b] and Emmanuel E. Attih[a]

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Cyathula prostrata is a herbal recipe used in traditional medicine for the treatment of chest troubles, dysentery, diarrhea, craw-craw, scabies, sexual disease, rheumatism, tumours and inflammatory conditions amongst many others. There is not yet any claim on the use of the plant as an antioxidant agent, hence the need for this study. The crude extract, fractions and isolates tested positive for the characteristic rapid TLC free-radical scavenging activity with β-carotene and DPPH reagents. The ethyl acetate fraction gave marginally similar antioxidant activity (IC_{50}) as the crude extract at 0.76 µg mL^{-1} while the activity demonstrated by the butanol fraction was equally marginal at 0.77 µg mL^{-1}. However, HOO-1 and HOO-2 gave moderate activity at 0.53 µg mL^{-1} and 0.56 µg mL^{-1} respectively which were comparably better than the antioxidant activity obtained with vitamin E and A at 0.60 µg mL^{-1} and 1.11 µg mL^{-1} respectively. Furthermore, vitamin C recorded an IC_{50} of 0.49 µg mL^{-1} which was comparatively better than the activity given by either HOO-1 or HOO-2. The antioxidant activities given by the extract, fractions, HOO-1 and HOO-2 were instructive as the phytochemical screening of the *C. prostrata* indicated the presence of terpenes, flavonoids and tannins which have been reported in previous studies to exhibit antioxidant activities. The results of the antioxidant assays have revealed a novel potential for the use of *C. prostrata* as an antioxidant agent. Hence, it is proposed that the mechanism of action of the antioxidant activity obtained especially with HOO-2 could have proceeded in the same way (a 2H stabilized resonance) as that of the antiscurbitic activity of vitamin C in literature.

Corresponding Authors
Tel: +2347038916740
E-Mail: wale430@yahoo.co.uk, olawaleoladimeji@hotmail.com
[a] Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy, University of Uyo, Uyo, Nigeria.
[b] Department of Pharmaceutical Chemistry, Faculty of Pharamceutical Sciences, University of Benin, Benin, Nigeria

INTRODUCTION

Free-radical oxygenated species (FROS) are believed to be responsible for inflammations. Also, free-radical damage and oxidative stress have become major health issues in recent years.

These chemical species have been implicated in many diseases and degenerative conditions such as Alzheimer's and Parkinson's diseases, heartdisease, stroke, cancer, pancreatitis, laryngitis, asthma, gastritis, dermatitis, hay fever, rheumatoid arthritis, wounds, atherosclerosis, emphysema, vitamin deficiencies, lung dysfunction, skin lesions, radiation injuries, premature aging and diabetes amongst many others.[1-12] *Cyathula prostrata* is employed in folklore medicine in the treatment and management of dyspepsia, scabies, craw-craw, diarrhea, dysentery, cholera, itch, ringworm, coughs, leprosy, sores,[13-18] articular arthritis, rheumatism,shingles, wounds, ulcers, inflammations[19-22] and sexually transmitted diseases.[23]

Though, its use in the treatment and management of inflammatory conditions is well known but there is yet any claims on its use as an antioxidant agent. Hence, the tests for free-radical scavenging (antioxidant) activity were considered relevant.

MATERIALS AND METHODS

The fresh aerial parts of *C. prostrata* (L.) Blume were collected in the month of July, 2011 on a farmland in Itak Ikot, Ikono Local Government Area, Akwa Ibom State, Nigeria. The authentication by comparison was done with herbarium samples of the Forestry Research Institute of Nigeria (FRIN) and the National Institute of Horticulture (NIHORT), both at Ibadan, in Oyo State, Nigeria. A voucher specimen of the plant (No H92) was deposited in the herbarium of the Faculty of Pharmacy, University of Uyo, Nigeria.

Extraction and processing

The plant was air-dried and powdered in an electric mill. The resultant coarse powder was then extracted with cold 96 % aqueous ethanol at room temperature (27± 2 ^{0}C) for 72 h. The filtrate was evaporated to dryness *in-vacuo* on a rotary evaporator (Buchi CH-920, Laboratorium Technic, Flawk/SG, Switzerland) and then stored in an amber bottle. Also, the aqueous extract of the plant was partitioned with organic solvents of increasing polarities namely, hexane, chloroform, ethyl acetate and butanol.The resultant mixtures were then bulked separately to obtain the hexane (3A), chloroform (3B), ethyl acetate (3C) and butanol (3D) fractions, respectively, which were then evaporated to dryness *in-vacuo* and then stored in a refrigerator at –4 ^{0}C prior to the antioxidant tests.

Chromatography

The ethyl acetate fraction (3C) was put through a combination of thin-layer, column and preparative chromatographies using silica-gel 254 (Sigma, USA) to obtain the isolates HOO-1 and HOO-2.

Initial rapid thin-layer chromatographic assays

β-Carotene assay

β-Carotene is a lipid soluble antioxidant which protects cell membranes from lipid peroxidation[24,25] hence, its selection in the screening of the plant for the initial free-radical scavenging activity.

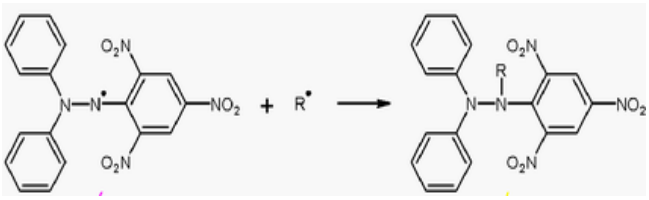
This model involves the oxidation of linoleic acid (an unsaturated fatty acid) by Reactive Oxygen Species (ROS). The products formed will then initiate the carotene oxidation which leads to discolouration.[26,27]

2,2-Diphenyl-1-picryhydrazyl hydrate (DPPH) assay

This assay is based on the principle of reduction. The purple color of the methanolic solution of DPPH is bleached when it accepts hydrogen or electrons from extract/ fraction/ isolate/standard antioxidant drug.The crude ethanolic extract, fractions, HOO-1 and HOO-2 were tested. The tests were carried out by developing the samples in ethyl acetate:methanol (1:2) in duplicates. Ascorbic acid (Emzor, Nigeria) was spotted along to serve as positive control. One chromatogram was sprayed with 0.1% w/v methanolic solution of β-carotene (Sigma, USA) while the other was sprayed with 0.1% w/v methanolic solution of DPPH (Sigma, USA). The plates were irradiated with ultra-violet light at λ_m 366nm for 15 minutes. Spots which appeared white on a bleached background[7,28-30] or white against a purple background[29,31-33] were taken as evidence of positive tests indicating antioxidant activity.

Spectrophotometric determination of antioxidant activity using DPPH reagent

Substances capable of donating electrons or hydrogen atoms (free radical scavengers) are able to convert the purple-colored DPPH radical (2,2-diphenyl-1-picryl-hydrazyl hydrate) to its yellow-colored non-radical form (1, 1-diphenyl-2-picrylhydrazine).[33,34] This reaction can be monitored by spectrophotometry.



Scheme 1. DPPH + R* (free-radical scavenger) = DPPH-R (Reduced DPPH)

This is the most widely reported method of screening for antioxidant activity in plants.[33-39] Hence, the antioxidant activity of *C. prostrata* was determined using the stable DPPH radical reagent.

Preparation of calibration curve for DPPH reagent

DPPH (4 mg) was weighed out and dissolved in methanol (100 mL) to produce a stock solution (0.004 % w/v). Serial dilutions were done to obtain the following concentrations; 0.0004, 0.0008, 0.0012, 0.0016, 0.0020, 0.0024, 0.0028, 0.0032 and 0.0036%w/v. The absorbance of each of the sample was obtained at λ_m 512nm[7,28-30] using ultra-violet spectrophotometer (Model No 3625, Unicam, England). A solution of methanol without DPPH was used as the blank for each of the determinations.Hence, the calibration curve for the DPPH reagent was prepared.

Determination of the antioxidant activity of crude extract, fractions, HOO-1 and HOO-2

2 mg of the crude ethanolic extract (2A), fractions (3A, 3B, 3C, 3D), HOO-1 and HOO-2 were separately dissolved in 50 ml of methanol. Serial dilutions were done to produce the following concentrations; 0.0008 mg mL^{-1}, 0.0016 mg mL^{-1} and 0.0024 mg mL^{-1} using methanol. 5 ml of each concentration was incubated with 5ml of 0.004 % w/v methanolic DPPH solution for optimal analytical accuracy.[28] After an incubation period of 30 minutes in the dark at room temperature (25 ± 2 ^{0}C), observation was made for a change in the color of mixture from purple to yellow.[33,34] The absorbance of each of the test samples was then taken at λ_m 512nm.[7,28-30,38] The Radical Scavenging Activity (RSA %) or Percentage Inhibition (PI %) of free radical DPPH was thus calculated: [34]

$$RSA\,(\%)(PI,\%) = 100\,\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}}$$

where

A_{blank} is the absorbance of the control reaction (DPPH solution without the test sample and

A_{sample} is the absorbance of DPPH incubated with the extract/ fraction/isolate/standard antioxidant drug.

Extract/fraction/isolate/standard antioxidant drug concentration providing 50 % inhibition (IC_{50}) was calculated using a graph of inhibition percentage against the concentration of the extract/fraction/isolate/standard antioxidant drug.[34,40,41]

DPPH assay of standard antioxidant drugs

Standard antioxidants namely, vitamin A (Fidson, Nigeria), vitamin C (Emzor, Nigeria) and vitamin E (Neimeth, Nigeria) were used. While vitamin C was in a tablet dosage form, vitamins A and E were formulated as gelatine capsules. The estimated weight of the formulations containing 2mg of the standard antioxidant drugs were determined by proportionality and then diluted. Methanol was used to dissolve vitamin C, while n-hexane was used to dissolve vitamins A and E. Thus, methanolic and n-hexane solutions of 0.004 % (w/v) DPPH were used for incubation of vitamin C, vitamin A and E respectively for 30 minutes. The absorbance values for the drugs were obtained at wavelength at λ_m 512nm and the IC_{50} determined.

RESULTS AND DISCUSSION

Collection, extraction and processing of plant

The plant was identified, authenticated and collected observing basic guidelines of plant collection. Also, the rules governing extraction and processing of extracts were kept, thus preventing any changes to the chemical composition of the crude extract.[42,43] Previous studies on the crude extracts revealed the presence of saponins, tannins, flavonoids, terpenes and cardiac glycosides while alkaloids, anthraquinones and cyanogenic glycosides were absent.[44-46]

Secondary metabolites such as saponins, cardiac glycosides, alkaloids, tannins and flavonoids have demonstrated in several previous studies[47-55] to be responsible for the cure or management of many ailments caused by microbes and different kinds of disease conditions in the ethno-medicine of plants.

Chromatography

The antimicrobial screening of the extract and fractions showed that the antimicrobial activity was most pronounced in the ethyl acetate fraction. Hence, the antimicrobial constituents of the crude extract resided largely in the ethyl acetate fraction, being the most active.

In addition, the ethyl acetate fraction extracted the largest amount of material. Consequently, silica-gel 254 chromatographic separation of the ethyl acetate fraction afforded HOO-1 and HOO-2.[45,46]

Rapid thin-layer chromatographic Analysis for antioxidant activity

The extract, fractions, HOO-1, HOO-2 and ascorbic acid gave white spots on bleached background when the chromatogram was sprayed with methanolic solution of β-carotene reagent.

The white spots (irrespective of initial spotted color) and the bleached background observed are pieces of evidence of carotene oxidation (discoloration). Also, the extract, fractions, HOO-1, HOO-2 and ascorbic acid showed white spots on a purple background when reacted with DPPH reagent.

The observed white spots (irrespective of initial spotted color) are evidence of the reduction of the DPPH reagent by the by free-radical scavenger in the samples.

Spectrophotometric determination of antioxidant activity

Preparation of calibration curve

A calibration curve was prepared for the DPPH radical reagent by measuring its absorbance at different concentrations. DPPH reagent obeys the Beer-Lambert law at concentrations of 50-100 μM.[31] The Beer-Lambert Law is the basis of absorption spectrophotometry.

Table 1. Preparation of calibration curve of methanolic solution of DPPH reagent

Concentration (%, w/v)	Absorbance, λ_{max} (512nm)
0.0004	0.065
0.0008	0.131
0.0012	0.191
0.0016	0.227
0.0020	0.264
0.0024	0.332
0.0028	0.373
0.0032	0.446
0.0036	0.518
0.0040	0.553

Therefore, a plot of absorbance against concentration for a cell of unit thickness (1cm) should give a straight line passing through the origin.[56,57] It was observed that a strict proportionality existed between the absorbance and concentration. Hence, a regression line which passed through the origin was obtained. The absorbance of the DPPH solution increased as the concentration increased as can be seen in **Table 1** and **Figure 1**.

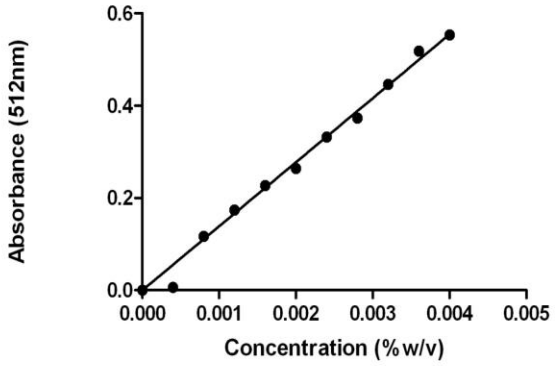


Figure 1. Calibration curve of methanolic solution of DPPH reagent.

Furthermore, the regression line buttresses this observation with a correlation factor of 0.99. Hence, the calibration curve obtained was used to correctly extrapolate subsequent concentrations of residual DPPH free radicals during the antioxidant test. Thus, the curve displayed in **Figure 1** confirms the purity, integrity and suitability of the DPPH reagent for the antioxidant assay.

Determination of the antioxidant activity of crude extract, fractions, HOO-1, HOO-2, vitamins A, C and E

The reduction of the DPPH radical was determined by measuring its absorption at a wavelength of λ_m 512 nm. It was observed that the absorbance of DPPH decreased as the concentration of added free radical scavenger (extract/fraction/isolate/standard antioxidant drug) increased which suggested that the DPPH reagent was being reduced. The results of the reduction are as presented in **Table 2**.

Table 2. Absorbance of test samples incubated with DPPH at different concentrations

Sample	Absorbance, λ_{max} (512 nm)		
	0.0008 mg mL^{-1}	**0.0016 mg mL^{-1}**	**0.0024 mg mL^{-1}**
2A	0.269	0.257	0.237
3A	0.299	0.299	0.304
3B	0.305	0.303	0.297
3C	0.268	0.259	0.244
3D	0.269	0.258	0.245
HOO-1	0.135	0.115	0.092
HOO-2	0.164	0.142	0.113
Vitamin A	0.292	0.257	0.243
Vitamin C	0.115	0.092	0.072
Vitamin E	0.154	0.154	0.154

Key: 2A=crude ethanolic extract of *Cyathula prostrata* 3A=hexane fraction; 3B=chloroform fraction; 3C=ethyl acetate fraction; 3D=butanol fraction; HOO-1=ethyl hexadecanoate (ethyl palmitate); HOO-2=7,9-di-tert-butyl-1-oxaspiro[4,5]deca-6,9-diene-2,8-dione.

The radical scavenging activity (RSA %) or percentage inhibition (PI, %) and the IC_{50} values of extract, fractions, isolates and standard antioxidant drugs were computed as **Table 3** shows. The RSA % is an indicator of the antioxidant activity of extract/fraction/isolate/ standard antioxidant drug.[33-38,58] The determined IC_{50} for the extract and ethyl acetate fraction were marginally similar at 0.76 µg mL^{-1}. Also, the antioxidant activity demonstrated by butanol fraction was equally marginal at 0.77 µg mL^{-1}. However, HOO-1 and HOO-2 gave moderate activity at 0.53 µg mL^{-1} and 0.56 µg mL^{-1}, respectively which were comparably better than the antioxidant activity obtained with vitamin A at 1.11 µg mL^{-1}. Vitamin C recorded an IC_{50} of 0.49 µg mL^{-1} which was comparatively better than the activity given by either HOO-1 or HOO-2 as can be seen in **Table 3.**

Table 3. Radical scavenging activity (percentage inhibition) of samples at different concentrations and IC_{50} of samples (blank absorbance of 0.004 w/v % methanolic DPPH reagent: 0.553)

Sample	RSA % (PI %)			IC_{50} mg mL^{-1}
	0.0008 mg mL^{-1}	**0.0016 mg mL^{-1}**	**0.0024 mg mL^{-1}**	
2A	51.28	53.53	57.22	0.76
3A	45.91	45.91	45.10	-
3B	44.92	45.26	46.38	-
3C	51.52	53.18	55.86	0.76
3D	51.28	53.42	55.75	0.77
HOO-1	75.53	79.29	83.30	0.53
HOO-2	70.29	74.31	79.53	0.56
Vitamin A	47.12	53.51	56.26	1.11
Vitamin C	79.21	83.34	86.92	0.49
Vitamin E	65.45	68.24	72.12	0.60

Key: Refer to Table 2; RSA % (PI %)=radical scavenging activity (percentage inhibition); IC_{50}=concentration at which 50 % of DPPH is scavenged or inhibited.

The antioxidant activities given by the extract, fractions, HOO-1 and HOO-2 were not surprising because the phytochemical screening of the *C.prostrata* indicated the presence of terpenes, flavonoids and tannins.[44-46] These classes of compounds have been reported in previous studies to exhibit antioxidant activities.[39,59-66] The results of the antioxidant assays have revealed a novel potential of *C. prostrata* as an antioxidant agent. A closer examination of the chemical structures of vitamin C and HOO-2 indicates some striking similarities between the two chemical entities as presented in **Figures 3** and **5**. There is a lactone ring common to both chemical substances. The mechanism of action of the antiscorbutic activity of vitamin C shows that there exists a 2H (2 hydrogen atom) stabilized resonance between the ascorbic acid and the dehydroascorbic acid isomers as reflected in **Figure 4.**[56,57]

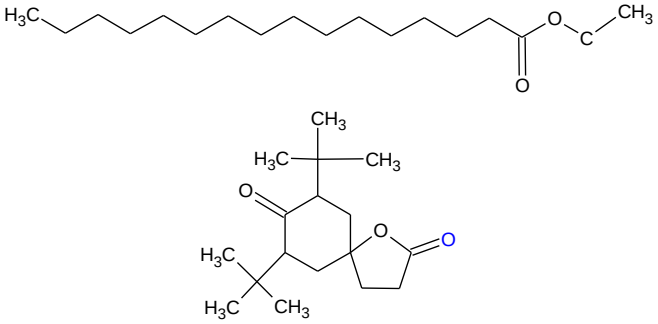


Figure 2. Chemical structures of HOO-1 (ethyl-hexadecanoate) and HOO-2 (7,9-di-tert-butyl-1-oxaspiro[4,5]deca-6,8-diene-2,8-dione

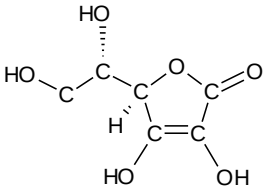


Figure 3. The Structure of Vitamin C

HOO-2 gave an antioxidant activity of 0.56 µg mL^{-1} which compare favourably with activity given by vitamin C at 0.49 µg mL^{-1}. Hence, it is proposed that the mechanism of action of the antioxidant activity obtained with HOO-2 might have proceeded in the same way (a 2H stabilized resonance) as that of vitamin C presented in **Figure 5**.

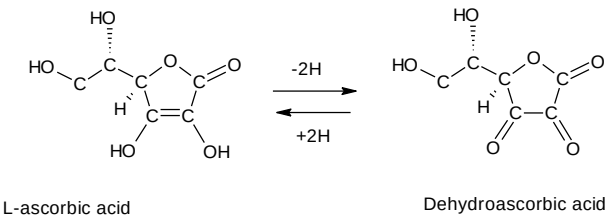


Figure 4. Mechanism of the antiscorbutic activity of Vitamin C

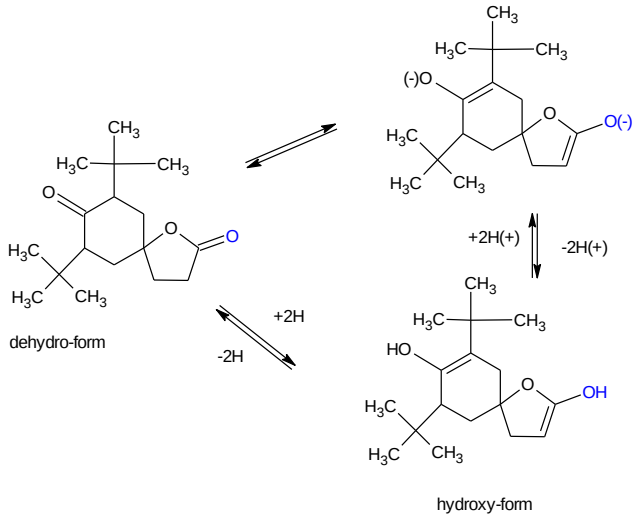


Figure 5. Proposed mechanism of the anti-oxidant activity of HOO-2 (7,9-di-tert-butyl-1-oxaspiro[4,5]deca-6,9-diene-2,8-dione).

Also, the importance of the radical scavenging ability of certain phytochemical compounds have found useful applications in the extension of shelf-life and control of deterioration of fatty foods, nutriceuticals and spices.[67-69]. Apart from the DPPH assay, other methods for determining the antioxidant activity of plants include the hydrogen peroxide, nitric oxide, conjugated diene, superoxide, phosphomolybdenum, peroxynitrile and xanthine oxidase assay methods amongst many others.[26,27]

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