



## ORIGINAL ARTICLE

# Novel Anti-oxidant Activity of *Cyathula prostrata* (L.) Blume

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### ABSTRACT

*Cyathula prostrata* is a herbal recipe used in traditional medicine for the treatment of chest troubles, dysentery, diarrhea, crawl-crawl, scabies, sexual disease, rheumatism, tumours and inflammatory conditions amongst many others. To date, there is yet any claims on the use of the plant as an anti-oxidant agent, hence this need for this study. The crude extract, fractions and isolates tested positive for the characteristic rapid TLC free-radical scavenging activity with  $\beta$ -carotene and DPPH reagents. The ethyl-acetate fraction gave marginally similar anti-oxidant activity ( $IC_{50}$ ) as the crude extract at 0.76  $\mu\text{g/ml}$  while the activity demonstrated by the butanol fraction was equally marginal at 0.77  $\mu\text{g/ml}$ . However, HOO-1 and HOO-2 gave moderate activity at 0.53  $\mu\text{g/ml}$  and 0.56  $\mu\text{g/ml}$  respectively which were comparably better than the anti-oxidant activity obtained with vitamins E and A at 0.60  $\mu\text{g/ml}$  and 1.11  $\mu\text{g/ml}$  respectively. Furthermore, vitamin C recorded an  $IC_{50}$  of 0.49  $\mu\text{g/ml}$  which was comparatively better than the activity given by either HOO-1 or HOO-2. The anti-oxidant 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 anti-oxidant 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 anti-oxidant activity obtained especially with HOO-2 could have proceeded in the same way (a 2H stabilized resonance) as that of the antiscorbic activity of vitamin C in literature.

**Keywords:** Free-radical; anti-oxidant;  $\beta$ -carotene; DPPH; *C. prostrata*.

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### INTRODUCTION

Free-radical oxygenated species (FROS) are believed to be responsible for inflammations, free radical damage and oxidative stress which 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, heart disease, 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], [2], [3], [4], [5], [6],[7], [8], [9],[10],[11],[12]. *Cyathula prostrata* is employed in folklore medicine in the treatment and management of dyspepsia, scabies, crawl-crawl, diarrhea, dysentery, cholera, itch, ringworm, coughs, leprosy, sores [13], [14], [15],[16], [17], [18], articular arthritis, rheumatism, shingles, wounds, ulcers, inflammations [19], [20], [21], [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 claim on its use as an antioxidant agent. Hence, the tests for free-radical scavenging (antioxidant) activity were considered relevant.

### MATERIALS AND METHODS

#### *Plant Collection and Authentication*

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 plant was identified by Dr. (Mrs) M. Bassey of the Department of Botany and Ecological Studies, University of Uyo, 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 ( $27 \pm 2^\circ\text{C}$ ) for 72h. The filtrate was evaporated to dryness *in-vacuo* on a rotary evaporator (Buchi H-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, thyl- 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  $-4^\circ\text{C}$  prior to the anti-oxidant 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*

*$\beta$ -carotene Assay*

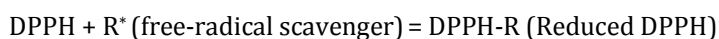
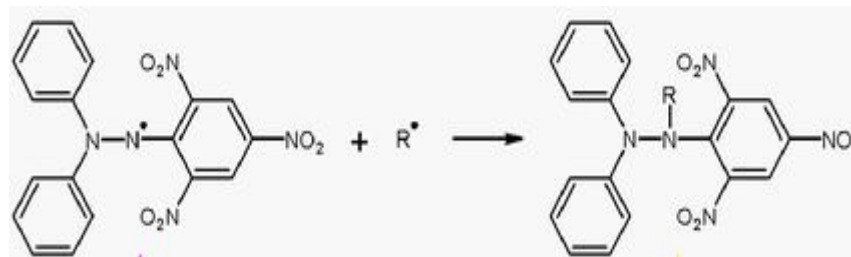
$\beta$ -carotene is a lipid soluble antioxidant which protects cell membranes from lipid peroxidation [24], [25] hence, its selection in the screening of the plant for the initial free-radical scavenging activity. This model involves the oxidation of linoleic acid (an unsaturated fatty acid) by Reactive Oxygen Species (ROS). The products formed will then initiate the carotene oxidation which leads to discoloration [26], [27].

*2, 2-Diphenyl-1-picrylhydrazyl hydrate (DPPH) Assay*

This assay is based on the principle of reduction. The purple colour of the methanolic solution of DPPH is bleached when it accepts hydrogen or electrons from extract/ fraction/ isolate/ standard anti-oxidant 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  $\beta$ -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  $\lambda_m$  366nm for 15 minutes. Spots which appeared white on a bleached background [7], [28],[29], [30] or white against a purple background [31], [32], [33] were taken as evidence of positive tests indicating anti-oxidant activity.

*Spectrophotometric Determination of Anti-oxidant Activity Using DPPH Reagent*

Substances capable of donating electrons or hydrogen atoms (free radical scavengers) are able to convert the purple-coloured DPPH radical (2, 2-Diphenyl-1-picrylhydrazyl hydrate) to its yellow-coloured non-radical form (1, 1-Diphenyl-2-picryl hydrazine) [33], [34]. This reaction can be monitored by spectrophotometry. This is the most widely reported method for anti-oxidant activity in plants [33], [34], [35], [36], [37], [38], [39]. Hence, the anti-oxidant activity of *C. prostrata* was determined using the stable DPPH radical reagent.



*Preparation of Calibration Curve for DPPH Reagent*

DPPH (4mg) was weighed out and dissolved in methanol (100ml) 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  $\lambda_m$  512nm [7], [28], [29], [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

2mg 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; 0.0008mg/ml, 0.0016mg/ml and 0.0024mg/ml using methanol. 5ml 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 ( $25 \pm 2^{\circ}\text{C}$ ), observation was made for a change in the colour of mixture from purple to yellow [33], [34]. The absorbance of each of the test samples was then taken at  $\lambda_{\text{m}}$  512nm [7,28, 29, 30, 38]. The Radical Scavenging Activity (RSA %) or Percentage Inhibition (PI %) of free radical DPPH was thus calculated:

$$\text{RSA\% (PI \%)} = [(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100 \quad [34].$$

$A_{\text{blank}}$  is the absorbance of the control reaction (DPPH solution without the test sample and  $A_{\text{sample}}$  is the absorbance of DPPH incubated with the extract/ fraction/ isolate/ standard anti-oxidant drug.

Extract/fraction/isolate/standard anti-oxidant drug concentration providing 50% inhibition ( $\text{IC}_{50}$ ) was calculated using a graph of inhibition percentage against the concentration of the extract/ fraction/ isolate/standard anti-oxidant drug [34], [40], [41].

*DPPH Assay of Standard Anti-oxidant Drugs*

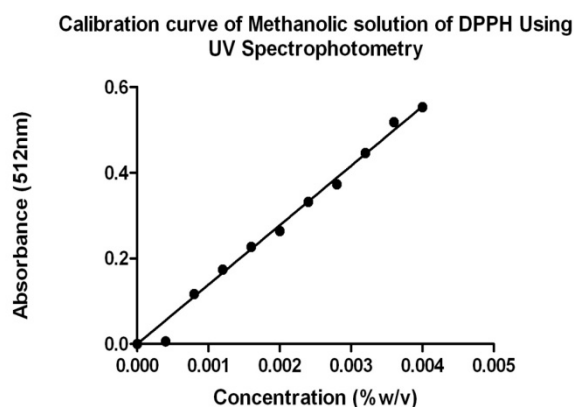
Standard anti-oxidants namely, vitamin A (Fidson, Nigeria), vitamin C (Emzor, Nigeria) and vitamin E (Neimeth, Nigeria) were used. While vitamin C was in a tablet dosage form, vitamins A and E were formulated as gelatine capsules. The estimated weight of the formulations containing 2mg of the standard anti-oxidant 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  $\lambda_{\text{m}}$  512nm and the  $\text{IC}_{50}$  determined.

## RESULTS AND DISCUSSION

**Table 1:** Preparation of Calibration Curve of Methanolic Solution of DPPH Reagent  $\lambda_{\text{max}}$  (512nm)

Concentration (%w/v)	Absorbance
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



**Figure 1:** Calibration Curve of Methanolic Solution of DPPH Reagent.

**Table 2:** Absorbance of Test Samples Incubated with DPPH at Different Concentrations  
 $\lambda_{\max}$  (512nm)

Sample	Absorbance		
	0.0008mg/ml	0.0016mg/ml	0.0024mg/ml
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.

**Table 3:** Radical Scavenging Activity (Percentage Inhibition) of Samples at Different Concentrations and IC<sub>50</sub> of Samples

(Blank Absorbance of 0.004w/v % Methanolic DPPH Reagent: 0.553)

Sample	RSA% (PI %)			IC <sub>50</sub> ( $\mu$ g/ml)
	0.0008mg/ml	0.0016mg/ml	0.0024mg/ml	
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

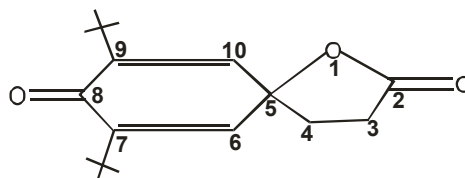
**Key:** Refer to Table 2;

RSA% (PI %) = Radical Scavenging Activity (Percentage Inhibition) ;

IC<sub>50</sub> = Concentration at which 50% of DPPH is Scavenged or Inhibited.



Ethyl Hexadecanoate (H001)



7, 9 - Di-tert-butyl-1-oxaspiro-(4,5)deca-6,9-diene-2,8-dione (H00 2)

**Figure 2:** Chemical Structures of HOO-1 and HOO-2.

#### 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], [45], [46].

Secondary metabolites such as saponins, cardiac glycosides, alkaloids, tannins and flavonoids have demonstrated in several previous studies [47], [48], [49], [50], [51], [52], [53], [54], [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  $\beta$ -carotene reagent. The white spots (irrespective of initial spotted colour) and the bleached background observed are pieces of evidence for carotene oxidation (discolouration). 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 colour) are evidence of the reduction of the DPPH reagent by the 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  $\mu$ M [31]. The Beer-Lambert Law is the basis of all absorption spectrophotometry. 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.

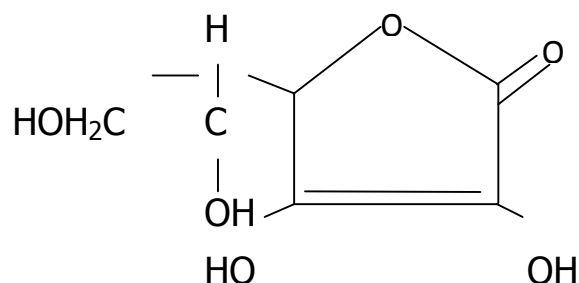
The absorbance of the DPPH solution increased as the concentration increased as can be seen in Table 1 and Figure 1. Furthermore, the regression line buttresses this observation with a correlation factor of 0.99. Hence, the calibration curve obtained was used to correctly extrapolate subsequent concentrations of residual DPPH free radicals during the anti-oxidant test. Thus, the curve displayed in Figure 1 confirms the purity, integrity and suitability of the DPPH reagent for the anti-oxidant 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  $\lambda_m$  512nm. It was observed that the absorbance of DPPH decreased as the concentration of added free radical scavenger (extract/fraction/isolate/standard anti-oxidant drug) increased which suggested that the DPPH reagent was being reduced. The results of the reduction are as presented in Table 2.

The Radical Scavenging Activity (RSA %) or Percentage Inhibition (PI %) and the  $IC_{50}$  values of extract, fractions, isolates and standard anti-oxidant drugs were computed as Table 3 show. The RSA % is an indicator of the anti-oxidant activity of extract/fraction/isolate/standard anti-oxidant drug [33],[34], [35], [36], [37], [38], [58]. The determined  $IC_{50}$  for the extract and ethyl-acetate fraction were marginally similar at 0.76  $\mu$ g/ml. Also, the anti-oxidant activity demonstrated by butanol fraction was equally marginal at 0.77 $\mu$ g/ml. However, HOO-1 and HOO-2 gave moderate activity at 0.53  $\mu$ g/ml and 0.56  $\mu$ g/ml respectively which were comparably better than the anti-oxidant activity obtained with vitamin A at 1.11 $\mu$ g/ml. Vitamin C recorded an  $IC_{50}$  of 0.49 $\mu$ g/ml which was comparatively better than the activity given by either HOO-1 or HOO-2 as can be seen in Table 3. The anti-oxidant 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], [45], [46]. These classes of compounds have been reported in previous studies to exhibit anti-oxidant activities [39], [59], [60], [61],[62],[63],[64], [65], [66]. The results of the anti-oxidant assays have revealed a novel potential of *C. prostrata* as an anti-oxidant agent. A closer examination of the chemical structures of vitamin C and HOO-2 indicates some striking resemblance between the two chemical entities as presented in Figures 3 and 5. There is lactone ring common to both chemical substances. The mechanism of action of the antiscorbic 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].

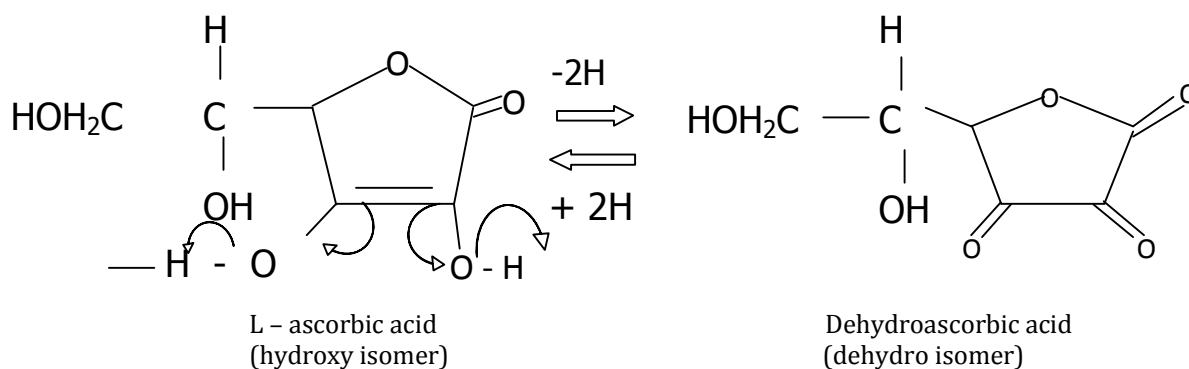
HOO-2 gave an anti-oxidant activity of 0.56  $\mu$ g/ml which compare favourably with activity afforded by vitamin C at 0.49 $\mu$ g/ml. Hence, it is proposed that the mechanism of action of the antioxidant activity obtained with HOO-2 could have proceeded in the same way (a 2H stabilized

resonance) as that of vitamin C presented in Figure 5. 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, nutraceuticals and spices [67], [68], [69]. Apart from the DPPH assay, other methods for determining the anti-oxidant activity of plants include the hydrogen peroxide, nitric oxide, conjugated diene, super oxide, phosphomolybdenum, peroxytrifluoromethane and xanthine oxidase assay methods and many others [26], [27].

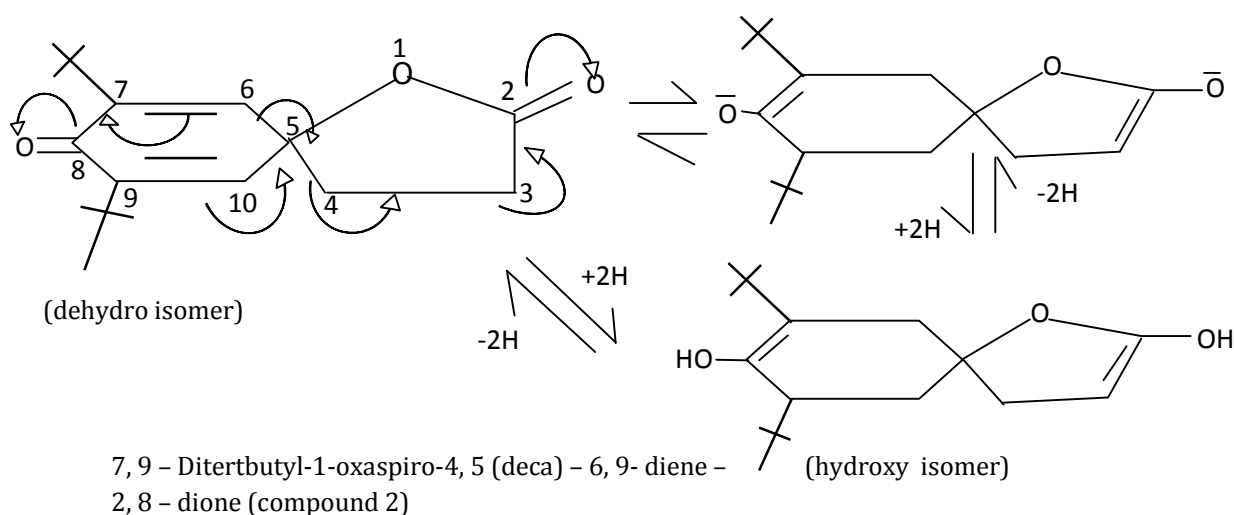


L - threo - 2, 3, 4, 5, 6 - pentahydroxy - 2 - hexenoic acid - 4-lactone (Ascorbic acid).

**Figure 3:** The structure of vitamin C



**Figure 4:** The mechanism of action of antiscorbic activity of vitamin C.



**Figure 5:** The proposed mechanism of action of the anti-oxidant activity of HOO-2 (7,9-Diterbutyl-1-oxaspiro-4,5 (deca)-6,9-diene-2,8-dione).

### CONCLUSION

The rapid TLC analyses (using  $\beta$ -carotene and DPPH reagents) and spectrophotometric determinations (DPPH reagent) carried out on *Cyathula prostrata* have revealed a novel potential of

the plant for use as an anti-oxidant agent in the fight against the incidence of free-radicals implicated in so many degenerative diseases.

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