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Evaluation of the antioxidant property of *Vernonia Cinerea* (L.) LESS. (Asteraceae) using 2,2-Diphenyl-1-Picrylhydrazine (DPPH) Assay Method

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Abstract

Vernonia cinerea (Asteraceae) is an annual, erect herb commonly found in roadside, open waste places, dry grassy sites and in perennial crops during plantation. It is located especially in different Asian countries such as India, Bangladesh and Nepal as well as in Nigeria. In Nigeria, the aqueous extract of the plant is drunk by the local people because it is to purify the blood. It is also used traditionally in the treatment of stomach aches, cough and eye problems. The ethanol extract of *Vernonia cinerea* (Asteraceae) was evaluated for its antioxidant property, using the DPPH assay method, and phytochemical constituents. An acute toxicity study, using animal model (mice). The result of the acute toxicity test of the extract on mice gave an LD₅₀ of 244.95mg/kg. phytochemical analysis revealed that the extract contained alkaloids, saponins, tannins, flavonoids, terpenes, glycoside, carbohydrates, steroids, phenols and esters. The concentration of the extract at which 50% of the DPPH radical was inhibited (IC₅₀) was seen to be 17.30µg/ml compared to the standard antioxidant (vitamin C-14.30 µg/ml). The study shows that *Vernonia cinerea* possess antioxidant property.

Keywords: *Vernonia cinerea*, Ethanol extract, Antioxidant properties.

Introduction

Today, there is revival of interest in the use of medicinal plants in the form of extract to develop relatively cheaper patentable chemical drugs with little or no side effects. In recent years there has been a great deal of attention towards the field of free radical chemistry (Lobo *et al.*, 2010) [9]. This development is producing a medical revolution that promises a new age of health and disease management (Aruoma, 2003) [1]. It is ironic that oxygen, an element indispensable for life, under certain situations has deleterious effects on the human body. Most of the potentially harmful effects of oxygen are due to the formation and the activity of chemical compounds called Reactive oxygen species. (Bagchik, 1998) [2]. Free radicals damage lipids, protein and DNA. This damage triggers a number of diseases in the human body.

Antioxidants are a wide range of substances or molecules that neutralize free radicals, preventing damage to living cells. Consumption of external sources (synthetic) of antioxidants can assist the body in coping with this oxidative stress (Lobo *et al.*, 2010) [9]. Researches are on-going, aimed at increasing the knowledge of antioxidant activities obtained from natural sources such as plants (Oke and Harmburger, 2002) This study is in support of this effort.

Vernonia Cinerea (L.) LESS

Vernonia cinerea, commonly called little iron weed is found along roadsides, open waste places, dry grassy sites and in plantations of perennial crops. The plant which belongs to the family Asteraceae is an erect, slender, rarely branching annual herb that grows up to 80cm tall. (Ashanul *et al.*, 2012) [3, 4].

In Akwa Ibom State, Nigeria the aqueous extract of *Vernonia cinerea* is taken as blood purifier. The infusion of the whole plant is given to patients with uncontrollable urine (Etukudo, 2003) [7].

Materials and Method

Collection and Identification of Plant

Fresh *Vernonia cinerea* (aerial parts) were collected in October, 2014 from a farmland in Uyo Local Government of Akwa-Ibom State. The plant was identified and authenticated as

Vernonia cinerea (Asteraceae) with the identification number, UUH 10K, by Dr. (Mrs) Margaret Basse of the Department of Botany and Ecological Studies, University of Uyo. The plants were sorted/graded, air dried and pulverized using a mortar and pestle for extraction.

Extraction

480g of the pulverized plant was marcerated, using 70% ethanol, in a marceration tank for 72 hours at room temperature (27 °C ± 2 °C). The tank was agitated three times daily to enhance the extraction process. The filtered extract was concentrated in a water bath at 40 °C. Upon complete drying, the ethanol extract was weighted and then stored in a refrigerator for subsequent use.

Phytochemical screening

The ethanol extract was screened phytochemically to identify the bioactive constituents. These tests were carried out using the standard methods of analysis by Trease and Evans (1989) [16] and Sofowora (2008) [15].

Acute Toxicity Study (LD₅₀)

18 albino mice of both sexes, weighing 14 - 20g, were obtained from the Animal House, Department of pharmacology and Toxicology, University of Uyo, Uyo. The mice were housed in animal cages under standard condition of light, humidity and temperature and fed on growers palletized feed with water.

The modified Lorke's method was used to determine the toxicity level of *Vernonia cinerea* ethanolic extract. The mice were fasted for 18 hours and were grouped in threes with each being assigned an identification number. In the initial phase, the first three groups of three mice each were administered three doses of the crude ethanol extract of the plant (100mg/kg, 200mg/kg, and 200mg/kg) intraperitoneally. The treated mice were monitored for 24 hours for mortality and general behaviour.

After 24 hours, the last three groups of mice were treated with the doses of 400mg/kg, 500mg/kg and 1000mg/kg of the plant extract based on the initial findings and were monitored for another 24 hours.

Test for Antioxidant Activity Using DPPH Method

Preparation of 0.004%w/v Methanolic and n-Hexane Solutions of DPPH

0.004%w/v methanolic solution of DPPH and 0.004%w/v n-hexane solution of DPPH were prepared by diluting 0.004g of DPPH in 100mls of methanol and n-hexane respectively. The absorbance of the methanolic solution and n-hexane solutions of DPPH were obtained at 517nm and recorded as blanks.

DPPH Assay

(a) DPPH Assay with crude Extract

100mg of the crude extract was dissolved in 50mls of methanol in volumetric flask to obtain the stock solution of 2mg/ml. The stock solution was serially diluted to give solutions with concentrations of 0.02mg/ml (20µg/ml), 0.04mg/ml (40µg/ml), 0.06µg/ml (60µg/ml), 0.08µg/ml (80µg/ml), and 0.1mg/ml (100µg/ml) respectively.

2mls of each of the diluted solution were incubated with 2mls of 0.004% methanolic DPPH at room temperature in a dark cupboard. After incubating for 30minutes, the absorbance was read for each concentrations against a blank at 517nm. The percentage inhibition (1%) of free radical DPPH was calculated as follows:

$$\% \text{ inhibition of DPPH radical} = \left[\frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right] \times 100$$

Where:

A_{blank} = Absorbance of the control reaction (DPPH without the test fraction)

A_{sample} = Absorbance of DPPH incubated fraction

(Guagrong *et al.*, 2008)

The concentration providing 50% inhibition (IC₅₀) was calculated using a graph of percentage inhibition against concentration of extract (Guagrong, 2008).

DPPH Assay with Standard Antioxidant

The above procedure was repeated for the standard antioxidants vitamin A, C and E. The tablet dosage form of vitamin C was used while vitamin A and E were in a gelatin capsule form. The estimated weight of the formulations that will provide a stock solution of 2mg/ml were determined by proportionality. 60mg of vitamin A was dissolved in 30mls of n-hexane, 100mg of vitamin C was dissolved in 50mls of methanol, and 100mg of vitamin E was dissolved in 50mls of n-hexane. Syringes were used to dilute as earlier described. However, while methanol was used to dilute vitamin C, n-hexane was used to dilute vitamins A and E.

Methanol solution of 0.004% DPPH was used to incubate vitamin C while n-hexane solution of 0.004% DPPH was used to incubate vitamins A and E for 30 minutes. The absorbance was then detected for all three vitamins each and used to calculate the percentage inhibition for each of the vitamins, the IC₅₀ for the three standards were also determined and the result compared with that of the plant extract.

Results and Discussion

Results

Extraction Yield

The percentage yield of the extract obtained from 480g of *Vernonia cinerea*, using 70% ethanol, is presented in table 1

Table 1: Percentage yield of the ethanolic extract of *vernonia cinerea*

Weight of Dried Pulvesied Plan Material (g)	Weight of Dried 70% Ethanolic Extract (g)	Percentage Yield (%)
480	14.11	2.24

$$\text{Percentage Yield} = \frac{\text{Weight of Dried Extract}}{\text{Weight of Dried Plan Material}} \times 100$$

Table 2: Results of the phytochemical screening

Constituents	Test	Inference
Alkaloids	Mayer's Reagent	+++
	Wagner's Reagent	+
Saponins	Fronthing	+++
	Emulsion test	+
	Sodium Bicarbonate	++
Tannins	Ferric chloride	+++
Flavonoids	Shinoda's test	+++
Terpenes	Leiberman's test	+++
Cardiac Glycoside	Keller-Killiiani test	+++
	Salkowski's test	+++
	Legal test	+++
Carbohydrate	Molisch test	++
	Barfoed's test	-
Steroids	Steroid Ring Test	++
Phenols	Ferric Chloride	+++
Esters	Hydrolytic test	+

Keys:

- (+++) = Present in High concentration
- (++) = Present in moderate concentration
- (+) = Present in low concentration
- (-) = Absent

Table 3: Results of the acute toxicity study

Dose (mg/kg)	Number of Dried Mice
100	0/3
200	0/3
300	3/3
400	3/3
500	3/3
1000	3/3

$$LD_{50} = \sqrt{AX B} = \sqrt{200 \times 300}$$

$$= 244.95 \text{mg/kg}$$

A = Maximum dose producing 0% mortality

B = Minimum dose producing 100% mortality

Table 4: The absorbance of the extract, vitamins A and E at different concentrations.

Concentration	Absorbance at 517		
	Extract	Vitamin A	Vitamin E
20	0.824	1.192	1.051
40	0.820	0.586	1.041
60	0.816	0.578	1.034
80	0.815	0.572	1.031
100	0.801	0.567	1.030

Table 5: The absorbance of vitamin C at different concentrations

Concentrations mg/ml	Absorbance at 517nm
10	1.670
20	0.838
30	0.185
40	0.154
50	0.142

Table 6: Percentage inhibition of DPPH radical at different concentrations and IC₅₀ of extract, vitamins A and E.

Concentration (µg/ml)	Percentage Inhibition		(%)
	Extract	Vitamin A	
20	57.37	30.67	38.86
40	57.58	65.88	39.44
60	57.79	66.37	39.85
80	57.84	66.73	40.02
100	58.56	67.02	40.08
IC ₅₀	17.30	31.50	-
Blank absorbance (517nm) of methanolic DPPH	1.933	1.719	1.719

Table 7: Percentage inhibition of DPPH radical at different concentrations and IC₅₀ of vitamin c

Concentration (µg/ml)	Percentage Inhibition Vitamin C (%)
10	35.78
20	67.78
30	92.87
40	94.08
50	94.54
Blank absorbance (517nm) of Methanolic DPPH	2.60

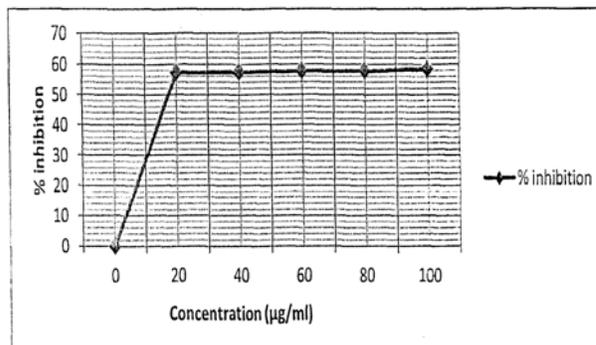


Fig 1: A Percentage Inhibition-Concentration Curve of the Ethnaol Extract of *Vernonia cinerea*

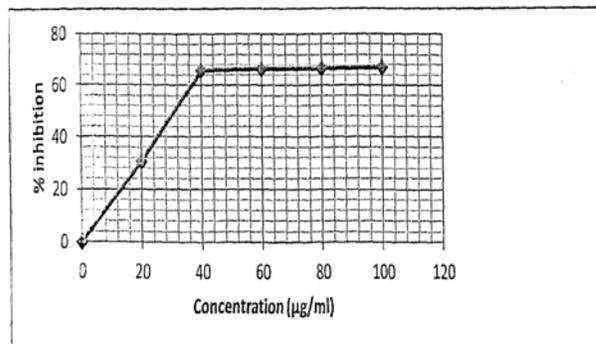


Fig 2: A Percentage Inhibition-Concentration Curve of Vitamin A

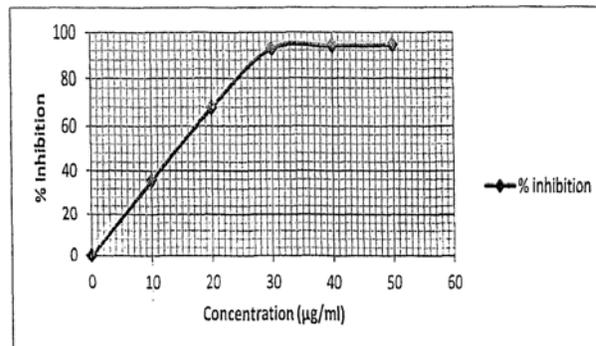


Fig 3: A Percentage Inhibition-Concentration Curve of Vitamin C

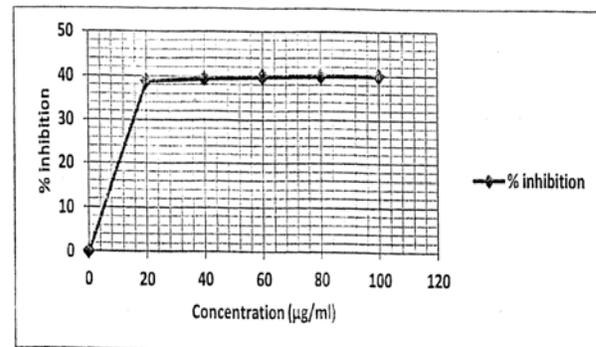


Fig 4: A Percentage Inhibition-Concentration Curve of Vitamin E

Discussion

The result of the phytochemical screening of the ethanol extract of *Vernonia cinerea* revealed the presence of alkaloids, saponins, tannins terpenes, glycosides, carbohydrates, steroids,

phenols and esters (table 2). Saponins are glycosides with foaming characteristics and hemolytic properties (Ahsanul *et al.*, 2012) [3, 4]. Tannins have been shown to constrict blood vessels, thus providing protective covering for wounds (Trease and Evans 2002) [17]. Flavonoids were present in abundance in the extract.

Flavonoids are phenolic substances that act in plant as antioxidants, antimicrobials, photoreceptors, visual attractors, feeding repellants and for light screening. The antioxidant activity of flavonoids is due to their ability to reduce free radical formation and to scavenge free radicals (Pietta, P G 2000) [12]. Terpenes are a class of compounds that comprise of essential oil. Cardiac glycosides have beneficial effect on the heart.

Carbohydrate provides energy and serves as cell membrane component. Most alkaloidal preparations are used as psychoactive substances and pain killers, (Trease and Evans, 2002) [17]. The LD₅₀ of the extract was 244.95 mg/kg. This shows that the extract has a good safety margin (table 3).

DPPH is a well known radical and a scavenger (trap) for other radicals. The delocalization of electron over the DPPH molecule is what gives rise to deep violet colour in solution, characterized by an absorption band at 517nm. Blios, 1958 recommended methanol as a good solvent for the preparation of DPPH solution. The extract and vitamin C were incubated in 0.004% w/v methanolic solution of DPPH because they are soluble in methanol.

Vitamins A and E were incubated in 0.004% w/v n-hexane solution of DPPH because the drugs are insoluble in polar solvents. The principle behind the DPPH assay of antioxidant activity is that when a solution of DPPH is mixed with that of a substrate that can donate electron or hydrogen atom, a reduced form of DPPH is formed with the loss of the normal violet colour of the methanolic DPPH solution to a colourless or pale yellow colour depending on the number of electrons taken up (Buijinsten *et al.*, 2001) [6].

The advantage of DPPH method of analysis is that DPPH radical is stable and need not be generated. This method allows DPPH to react slowly with the whole sample even with weak antioxidants. Prakash, 2001) [13].

Vitamins A, C and E were employed as standards antioxidants to check the correctness of the procedure and also to compare their antioxidant activities with that of the extract. Molyneux (2004) [11] recommended that results of the antioxidant efficiency should involve the use of a standard to avoid doubts concerning the correctness of the result in the direct determination of DPPH obtained from calibration curve (Leitao *et al.*, 2002) [8]. When vitamin C was incorporated with DPPH solution, the purple colour of the DPPH solution changed to pale yellow.

Vitamin A and the extract yielded little colour change during incubation. This shows that vitamin C has a higher antioxidant activity than vitamin A or the extract. As the concentration of the extract, vitamin A or E increased from 20 to 100mg/ml their absorbance decreased from 0.824 to 0.801, 1.192 to 0.567, and 1.051 to 1.030 respectively (table 4).

Similarly as the concentration of vitamin C increased from 10 to 50 mg/ml its absorbance decreased drastically from 1.670 to 0.142(table 5).

The higher the concentration of the antioxidant the higher the speed with which the antioxidant reacts with the DPPH. This results in increased antioxidant activity and increased percentage inhibition of radical leading to low absorbance and low IC₅₀ (Buijinsten *et al.*, 2001) [6].

The extract of *Veronia Cinerea* gave an IC₅₀ of 17.30mg compared to vitamin C (14.30mg/ml) and vitamin A (31.50mg/ml). The IC₅₀ of vitamin E could not be detected. This could be as a result of low concentration of the vitamin (tables 6 and 7).

A lower IC₅₀ value indicates a higher antioxidant activity (Guangrong *et al.*, 2008).

Conclusion

The results obtained from this study led to the following conclusions:

- (1) That the LD₅₀ of the ethanol extract of *Veronia Cinerea* on mice was 244.95mg/kg.
- (2) That the phytochemical constituents of *Veronia Cinerea* include alkaloids, saponins, tannins, flavonoids, terpenes, glycosides, carbohydrates, steroids, phenols and esters.
- (3) That *Veronia Cinerea* possess significant antioxidant property thus justifying the drinking of the aqueous extract of this plant as blood purifier in Nigeria.

Recommendation

- (1) Further research should be carried out on extracts of solvents other than ethanol to ascertain the extract that could give the highest antioxidant activity.
- (2) Other models should be used to assay the antioxidant activity of the plant because a good conclusion cannot be drawn based on the DPPH model alone.

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