

INVITRO AND INVIVO STUDIES ON THE ANTIOXIDATIVE ACTIVITIES, MEMBRANE STABILIZATION AND CYTOTOXICITY OF WATER SPINACH (*Ipomoea aquatica* Forsk) FROM IBAJI PONDS, NIGERIA.

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ABSTRACT : In this study, possible antioxidant activities membrane stabilizing potential and cytotoxicity of ethanol extract from water spinach (*Ipomoea aquatica* Forsk) leaf and stem were examined. 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical, nitric oxide radical, invivo antioxidant enzyme activity assay were carried out to evaluate the antioxidant potential of the extract. The antioxidant activity of the methanol extract increased in a concentration dependent manner. In DPPH radical scavenging assays the IC₅₀ value of the extracts were 33.188 and 672.376 (µg/ml) for the stem and leaf respectively. The plant inhibited the nitric oxide radicals generated from sodium nitroprusside with IC₅₀ of 142.52 and 156.99 (µg/ml) for the stem and leaf respectively as opposed to 0.0161 (µg/ml) for vitamin C. The total polyphenolic constituent could be contributory to the antioxidant activity observed. The plant extracts are less toxic (LC₅₀ = 160.8664 and 111.419 µg/ml) for the leaf and stem respectively when compared to the reference standard (potassium dichromate, LC₅₀ = 44.20 µg/ml). The membrane stabilizing activity of the extracts compare favourably with that of the standard anti-inflammatory drug used (indomethacin). The results obtained in the present study indicate that *Ipomoea aquatica* can be a good source of anti-inflammatory drug, relatively safe for consumption as utilized and some what a source of natural antioxidants agent.

Key words: *Ipomoea aquatica*, antioxidant activity, DPPH assay, membrane stabilization and cytotoxicity.

INTRODUCTION:

Plants have fed the world and cured its ills since time immemorial. The use of plant in curing and healing is as old as man himself. A vast knowledge of medicinal plant must therefore have accumulated. But most of this knowledge only exists as verbal tradition and only a fraction has got scientific basis till to date.

The use of plants products is increasing in many segment of the population¹. At present thousands of plant metabolites are being successfully used in the

treatment of variety of diseases. According to an estimate, 80% of the world's population rely upon plants for their medication². Since Nigeria have a vast resources of medicinal plants, the present study may be a significant way of making the best use of natural resources. The majority of our population, who are impoverished have to rely upon indigenous system of medication because of their inability to meet the cost of modern medicines. Moreover, the standardization of

herbal medicines has made them popular in many developed countries³.

Reactive oxygen species (ROS), which include free radicals such as superoxide anion radical (O_2^-), hydroxyl radical (OH) and non-free-radical species such as H_2O_2 and singlet oxygen (1O_2), are various forms of activated oxygen⁴⁻⁶. These molecules are exacerbating factors in cellular injury and in the aging process⁷. ROS have aroused significant interest among scientists. Their broad range of effects on biological and medicinal systems has been studied in many experimental investigations⁸. These reactive oxygen species play an important role in degenerative or pathological processes, such as aging⁹, Cancer, coronary heart diseases, Alzheimer's disease¹⁰⁻¹³, neurodegenerative disorders, atherosclerosis, Cataracts, and inflammation¹⁴. The use of traditional medicine is widespread, and plants are still a large source of natural antioxidants that might serve as leads for the development of novel drugs.

Again, the effect of anti-inflammatory drugs including herbal preparation on the stabilization of erythrocyte membrane exposed to hypotonic and heat has been studied extensively¹⁵. The erythrocyte membrane resemble lysosomal membrane as such, the effect of drugs on the stabilization of erythrocyte could be extrapolated to the stabilization of lysosomal membrane¹⁶.

The aquatic plant, water spinach (*Ipomoea aquatica Forsk*) grows uncultivated in ponds and slow flowing rivers in Ibaji Eastern Kogi State, Nigeria. It is long known as animal and aquatic organism feed, but recently, few individuals have found it a delicacy as it is being consumed as vegetable in the region. Water spinach is known to possess an insulin-activity according to indigenous medicine in SriLanka. Only a very few scientific studies have been conducted on its medicinal aspects. These include the inhibition of prostaglandin synthesis¹⁷ effects on liver disease¹⁸ constipation¹⁹.

This work was set out therefore to investigate membrane stabilizing activity, cytotoxicity of the plant as it is being discovered as important vegetable in Ibaji and to determine antioxidant potentials of the plant.

MATERIALS AND METHOD

PLANT MATERIALS.

Fresh water spinach (*Ipomoea aquatica Forsk*) was collected from Ibaji road, Idah, where the plant thrives very well. The plant material was washed with water to remove dirt and air-dried for two weeks. The dried plant material was pulverized using electric blender. Weighed portion of the sample was subjected to cold extraction.

SAMPLE PREPARATION

Cold extraction method was employed for the extraction. A portion (30g) of the powdered sample was weighed into a conical flask. Pure methanol (150ml) was added and left for 72 hours. The mixture was filtered and the filtrate was concentrated using rotary evaporator.

CHEMICALS

DPPH (2,2-diphenyl-1-picrylhydrazyl) (DPPH) and Griess reagent were purchased from sigma chemical company (Sigma Germany). Vitamin C used was a product of Glaxo Smithkline. Methanol, Folin-cio calteu reagent, potassium dichromate, ferric chloride, amyl alcohol and sodium nitroprusside were products of BDH. Tanic acid used was May and Baker product. The Glutathione peroxidase and superoxide dismutase kits were of the Randox laboratories, U.K.

POLYPHENOL COMPOSITION MEASUREMENT

The total phenol composition was measured utilizing the Folin-cioalceu reagent as described by²⁰. The method of Harbone²¹ was employed in the determination of the total flavonoid and anthocyanin content. The colorimetric method of Van-Burden and Robinson²² (1981) was employed in the determination of tannin content.

NITRIC OXIDE RADICAL INHIBITION ASSAY

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interact with oxygen to produce nitrite ions which were measured by Griess reaction^{23,24}. The reaction mixture (3ml) containing sodium nitroprusside (10mM) in phosphate buffered saline (PBS) and the varying concentration (1000,500, 250,125 and 62.5Ng/ml) of the extract and reference compound were incubated in a water bath at 25°C for 150 minutes. After incubation, 1.5ml of the reaction mixture was removed and 1.5 of Griess reagent was then added. The absorbance of the chromophore formed was evaluated using spectrophotometer at 546nm. Percentage inhibition of the nitric oxide radical generated was calculated using the formular

$$\left\{ \frac{(1-E)}{C} \times 100 \right\}$$

Where C = absorbance of the fully oxidized control

E = absorbance in the presence of extract

RAPID RADICAL SCAVENGING SCREENING

The method of Mensor²⁵ was followed in screening for the antioxidant property of the extracts. With the aid of capillary tube, stock solution (1mg/ml) of extracts were spotted on silica gel thin layer chromatographic (TLC) plate and developed with a solvent system of ethanol: methanol (90:10). After development, the chromatograms were dried and sprayed with a 0.3mM solution of the stable radical DPPH., Yellow spot formed against purple background were taken as positive results. The duration for the development of yellow colour indicated whether the antioxidant activity is strong or not.

MEASUREMENT OF DPPH FREE RADICAL SCAVENGING ACTIVITY

The free radical scavenging activities of the plant extracts were measured employing the modified method of Blois²⁶. A portion (1ml) each of the different concentrations (1000,500,250,125 and 62.5µg/ml) of extracts or standard (vitamin C) in a test tube was added 1ml of 0.3mM DPPH in methanol. The mixture was vortexed and then incubated in a dark chamber for 30 minutes after which the absorbance was measured at 517nm against a DPPH control containing only 1ml of methanol in place of the extract. Percentage scavenging activity was calculated using the expression: % scavenging activity = Absorbance of control – Absorbance of sample / Absorbance of control X 100.

INVIVO ANTIOXIDANT ENZYME ACTIVITY ASSAY

ANIMALS

Wister albino rats (male) were used for the study of the Crude extracts. The animals were kept at 27 ± 2°C, relative humidity 44 – 56% and light and dark cycles of 10 and 14 hours respectively for one week before and during the experiment. Animals were provided with standard diet (Mouse cubes – Top feed, Anyigba) and the food was withdrawn 18 – 24 hours before the start of the experiment and watered adlibitum. All the experiments were performed early in the morning according to the current guidelines for the care of the laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals²⁷.

ANIMAL GROUPING AND CRUDE EXTRACT ADMINISTRATION

Rats were divided into six (6) groups of four rats each. Group 1 (Control) animals were administered a single dose of water (1ml/kg b.w) orally, daily for 5 days and received liquid paraffin (1ml/kg b.w S.C), Group 2 (CCl₄) received water (1ml/kg b.w orally) once daily for 5 days and received CCl₄: liquid paraffin (1:1, 2ml/kg b.w., S.C). Test group animals (Group 3-6) were administered orally a dose of 200mg/kg b.w of methanol extracts in the form of aqueous suspension once daily.

The groups (4 and 6) animals were administered simultaneously CCl₄: lipid paraffin (1:1, 2ml/kg b.w, S.C) after 30 minutes. Animals were sacrificed after the last treatment. Blood samples were collected via cardiac puncture into EDTA bottles and refrigerated until used for the biochemical investigations.

ESTIMATION OF GLUTATHIONE PEROXIDASE (GPX) AND SUPER OXIDE DISMUTASE (SOD) ACTIVITY.

These two biochemical parameters, glutathione peroxidase (GPX)²⁸ and super oxide dismutase (SOD)²⁹

were assayed using assay kits (Randox laboratories limited, Ireland).

CYTOTOXICITY TO BRINE-SHRIMPS

Modified method of Solis³⁰ was used to determine the inhibitory activity on *Artemia salina* in Vial bottles. A portion (50µl) of the crude methanol extract in 0.25% Tween 80 – artificial sea water was added into each well (vial bottles) containing 10 newly hatched brine-shrimps in 50µl artificial sea water, then incubated at room temperature for 24 hours. All samples were repeated in 2 wells to make the overall tested organisms of 20 for each. The living brine-shrimps were counted under a hand magnifying lens. Same procedure was followed using potassium dichromate as the reference standard. Plot% lethality versus log concentration. Substituted y=50 in the resulted linear equation to obtain the X value. The antilog X was then the LC₅₀ (conc. of 50% lethality) value³¹.

MEMBRANE STABILIZING ACTIVITY ASSAY

The method of Sadique³² as modified by Oyedapo and Famurewa³³ and Oyedapo³⁴ was employed in the membrane stabilizing activity assay. The assay mixture consisted hyposaline (2ml), 1ml of 0.15M sodium phosphate buffer at pH 7.4. Varying volumes of drugs (2mg/ml) (0.0-1.0ml) and 2% (V/V) erythrocyte suspension in isosaline (0.5ml) were made up with isosaline to give a total assay volume of 4.5ml. The control was prepared as above except the drug was omitted; while drug control (4.5ml) lacked erythrocyte suspension. The reaction mixtures were incubated at 56°C for 30 minutes. The tube was cooled under running water followed by centrifugation at 5,000rpm. The supernatant were collected followed by reading of the absorbance of the released hemoglobin at 560nm. The percentage membrane stability was estimated using the expression: membrane stability =

$$\frac{100 - (100 \times (\text{drug test value} - \text{drug control value}))}{\text{Control value}}$$

STATISTICAL ANALYSIS.

Data are reported as the mean ± S.E.M of three determinations, IC₅₀ values for all the above experiments were determined by linear regression.

RESULTS AND DISCUSSION

Quantitative estimation of the phytochemical constituents of this vegetable/medicinal plant studied is as presented on table 1.

Table 1: Percentage phenolic constituents of *Ipomoea aquatica*

Plant parts	Flavonoids (%)	Tannin (%)	Phenol (%)	Anthocyanin
Stem extract	0.02 ± 0.014	0.24 ± 0.001	0.35 ± 0.07	1.77 ± 0.35
Leaf extract	0.29 ± 0.014	0.24 ± 0.02	0.31 ± 0.01	0.9 ± 0.02

Each value in the table was obtained by calculating the average of three determinations ± Standard Error of Mean (SEM).

Table 2: Rapid radical scavenging screening of *Ipomoea aquatica*

Plant parts	Reaction speed	Intensity of spots
Stem	Fast	+++
Leaf	Not very fast	++

Key: +++ = strong intensity (immediate reaction)
 ++ = intermediate intensity (1-5 minutes before colour develops)
 + = weak intensity of yellow coloration (15-30 minutes before colour develops)
 - = no yellow coloration

Table 3: DPPH radical scavenging activity of *Ipomoea aquatica*

Plant parts/standard	Concentration (µg/ml)	Log concentration	% Scavenging activity	IC ₅₀ µg/ml
Stem extract	1000	3.00000	76.97	33.188 ^a
	500	2.69897	71.19	
	250	2.39794	67.17	
	125	2.09691	60.56	
	62.5	1.79588	54.66	
Leaf extract	1000	3.00000	53.48	672.376 ^b
	500	2.69897	49.17	
	250	2.39794	42.69	
	125	2.09691	40.31	
	62.5	1.79588	34.72	
Vitamin C	1000	3.00000	96.95	0.0161 ^c
	500	2.69897	90.73	
	250	2.39794	89.57	
	125	2.09691	85.93	
	62.5	1.79588	85.11	

a. Linear equation: $y = 18.35X + 22.09$

b. Linear equation: $y = 14.74X + 8.321$

c. Linear equation: $y = 9.460X + 66.97$

Table 4: Nitric oxide radical scavenging activity of *Ipomoea aquatica*

Plant parts/standard	Concentration ($\mu\text{g/ml}$)	Log concentration	% Scavenging activity	IC ₅₀ $\mu\text{g/ml}$
Stem extract	1000	3.00000	92.85	142.52 ^a
	500	2.69897	86.64	
	250	2.39794	72.23	
	125	2.09691	49.12	
	62.5	1.79588	21.92	
Leaf extract	1000	3.00000	90.25	156.99 ^b
	500	2.69897	86.01	
	250	2.39794	71.01	
	125	2.09691	41.87	
	62.5	1.79588	21.72	
Vitamin C	1000	3.00000	96.95	0.0161 ^c
	500	2.69897	90.73	
	250	2.39794	89.57	
	125	2.09691	85.93	
	62.5	1.79588	85.11	

a. Linear equation: $y = 59.58X - 78.34$

b. Linear equation: $y = 60.19X - 82.17$

c. Linear equation: $y = 9.460X + 66.97$

Table 5: Effects of *Ipomoea aquatica* extracts on the activities of Glutathione peroxidase (GPX) and super oxide dismutase (SOD)

Group	Treatment	GPX (U/L)	SOD ($\mu\text{g/ml}$)
1.	Control	407.42 + 11.896	41 + 15.56
2.	CC ₄	368.95 + 11.896	18 + 16.97
3.	Stem extract	425.12 + 7.90	88 + 12.97
4.	Stem + CCl ₄	386.95 + 10.11	41 + 15.57
5.	Leaf extract	409.51 + 4.16	76 + 13.94
6.	Leaf + CCl ₄	375.95 + 8.41	41 + 10.53

Each value in the table was obtained by calculating the average of four replications \pm S.E.M.

Table 6: Inhibitory effects of Ipomoea aquatica crude extract on bruine-shrimps

Plant parts/standard	Concentration ($\mu\text{g/ml}$)	Log concentration	% Scavenging activity	LC ₅₀ $\mu\text{g/ml}$
Stem extract	1000	3.00000	60	111.419 ^a
	500	2.69897	55	
	250	2.39784	55	
	125	2.09691	50	
Leaf extract	1000	3.00000	65	160.864 ^b
	500	2.69897	60	
	250	2.39784	50	
	125	2.09691	50	
Potassium dichromate	1000	3.00000	100	44.20 ^c
	500	2.69897	80	
	250	2.39784	70	
	125	2.09691	70	

a. Linear equation: $y = 9.966X + 29.60$

b. Linear equation: $y = 18.27X + 9.688$

c. Linear equation: $y = 33.219X - 4.658$

Table 7: Membrane Stabilizing activity of crude methanol extracts of Ipomoea aquatic on human RBC subjected to heat and hypotonic stresses.

Plant parts/standard	Percentage membrane stabilization
Stem extract	43.2545 \pm 6.213
Leaf extract	69.77 \pm 4.20
Indomethacin	59.700 \pm 5.127

Values are the mean \pm S.E.M of three determinations.

In the present study, the antioxidative properties of *Ipomoea aquatica* leaf and stem were determined and compared using standard methods. The antioxidant activity of *Ipomoea aquatica* might be due to inactivation of free radicals or complex forming with metal ions, or combinations thereof. The antioxidative characteristics might be attributed to the presence of phytochemicals such as flavonoids, and other polyphenolic compounds. Table 1 shows the percentage polyphenolic constituents of the plant extracts. The stem extract posses high amount of these polyphenols compared to the leaf. These polyphenols have been known to show medicinal activity as well as exhibiting physiological activity³⁵. The compound such as flavonoid; which contain hydroxyls are responsible for the radical scavenging effects in plants³⁶.

DPPH RADICAL SCAVENGING ACTIVITY

The DPPH radical is considered to be a model of lipophilic radical. The radical scavenging activity of *I. aquatica* was determined from the reduction in absorbance at 517nm due to scavenging of stable DPPH free radicals. The positive DPPH test suggests that the samples are free radical scavengers (Tables 2, and 3). The scavenging effects of the stem and leaf extracts on the DPPH radical are shown in table 3. The stem extract had significant scavenging effects on the DPPH radical which increased with increasing concentration in the 62.5 – 1000 $\mu\text{g/ml}$ range. The stem extract had higher antioxidant capacity though not close to the standard used.

In the present study, the crude methanol extracts of *Ipomoea aquatica* were investigated for their inhibitory potential on nitric oxide production. Table 4 shows the percentage inhibition of nitric oxide generation by *Ipomoea aquatica* extracts. Nitric oxide radical scavenging activity is concentration dependent. The concentration of *Ipomoea aquatica* stem extract needed for 50% inhibition was 142.52 ($\mu\text{g/ml}$) which is better than the leaf extract. In vivo antioxidant activity was assayed by estimation of glutathione peroxidase (GPX) and superoxide dismutase (SOD) levels. The results of *Ipomoea aquatica* extracts on the activities of antioxidant enzymes are as illustrated on table 5. GPX and SOD contents increased in extract treated groups whereas CCl_4 – intoxicated groups have shown decrease in levels of enzyme activity compared to the control group.

To prevent damage to cellular components, there are numerous enzymatic antioxidant defenses designed to scavenge reactive oxygen species in the cell. These natural antioxidants are manufactured in the body, provide an important defense against free radicals., these enzymes include Glutathione peroxidase, catalase, and superoxide dismutase. Some of these enzymes are also present in plants. In plants, SOD isoenzymes are present in the cytosol and mitochondria. As presented on table 5, the stem extract showed highest activity of GPX and SOD respectively. CCl_4 is being used extensively to investigate hepatoprotective and antioxidant activity on various experimental animals³⁷. A major defense mechanism involves the antioxidant enzymes, including SOD, catalase and glutathione peroxidase which convert active oxygen molecules into non-toxic compounds. The toxic metabolite CCl_4 radical is produced which further reacts with oxygen to give trichloromethyl peroxy radical. Cytochrome P450 2E1 is the enzyme responsible for this conversion. This radicals bind covalently to the macromolecules and cause peroxidative degradation of lipid membrane of the adipose tissue.

In this present study, increase in the activity of this enzyme in the extract treated groups could suggest stabilization of plasma membrane as well as repair of hepatic tissue damage cause by CCl_4 . The significant increase in GPX and SOD content of the whole blood used suggested antioxidant activity of *Ipomoea aquatica* parts used. Thus, it can be inferred that the possible mechanism of antioxidant activity of the *Ipomoea aquatica* extracts may be due to their free radical scavenging activity which may be due to the presence of polyphenolic compounds in the extracts.

The results of the membrane stabilizing activities of the extracts are as presented on table 7. The results showed that the extracts are highly potent on human erythrocyte adequately protecting it against heat and hypotonic induced lyses. The activity was comparable to that of the standard anti-inflammatory drug (Indomethacin). The high membrane stabilizing activity of the leaf extract of *Ipomoea aquatica* could be attributable to the polyphenolic content. Earlier reports have shown that various herbal drugs are capable of

stabilizing the red blood cell membrane and exert anti-inflammatory activity^{32,38}.

The inhibitory effects on brine-shrimps of *Ipomoea aquatica* extracts are as presented on table 6. The extracts possessed very low cytotoxicity to brine-shrimps with LC_{50} of 160.864 ($\mu\text{g/ml}$) for the leaf and 111.419 ($\mu\text{g/ml}$) for the stem.

In this study, different antioxidative parameters were measured utilizing standard methods. These parameters include, DPPH radical scavenging, activity, nitric oxide radical inhibition, rapid radical scavenging screening, activity of antioxidant enzymes. The biosafety of the plant extract was assessed using brine-shrimps and its effect on the membrane stability. On the basis of the results obtained in the present study, we conclude that methanol extracts of *Ipomoea aquatica* has some amount of antioxidant capacity and it is relatively safe for the purposes utilized especially as new found food in Ibaji area of Nigeria. Anyway further studies are required to isolate the active components which are responsible for the antioxidant capacity and membrane stabilizing activity of water spinach.

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