Materials and Methods
MATERIALS AND METHODS

1) The Plant Materials

The leafy vegetables chosen for the study are Enydra fluctuans Lour.; Ipomoea aquatic Forssk; Lagenaria siceraria (Molina) Standl.; Cucurbita maxima Duchesne.; Trichosanthes dioica Wall.; Paederia scandens (Lour.) Merr.; Chenopodium album L.; Amaranthus blitum L.;; Glinus oppositifolius(L.) Aug. DC.; Moringa oleifera Lam. The samples were authenticated by the CNH, Botanical Survey of India (Vide letter no.CNH/120/2011/Tech II/607 and CNH/125/2011/Tech II/663).

II) Chemicals:

DPPH, ABTS + were purchased from Sigma Aldrich Chemical Company, USA; Standard rutin, quercetin from Sigma Chemical Co. St. Louis, MO; Ascorbic acid, trichloroacetic acid (TCA), ferric chloride (anhydrous) were supplied by E. Merck, India Limited. Potassium persulfate, Folin Ciocalteau’s phenol reagent, gallic acid, potassium ferricyanide, ethylenediamine tetra acetic acid disodium salt (EDTA) were purchased from SRL (Sisco Research Laboratory, Mumbai, India). All other chemicals and solvents were of analytical reagent grade, unless otherwise mentioned.
Extraction and quantification:

The leafy vegetables were purchased from local market. They were derooted, washed thoroughly in tap water, rinsed in distilled water, cut into small pieces and dried in hot air oven at 50±2°C. The dried LVs were finely powered in the grinder. 250 mg of the powder was weighed and extracted with 80% methanol in a homogenizer (REMI) and filtered. The content was decanted through a filter paper and the residue is again homogenized with 80% methanol. The extracts were pooled and evaporated in a rotary evaporator till free of solvent. The polyphenols obtained were dissolved in 10ml of distilled water.

Determination of polyphenols:

Chemicals: Folin Ciocalteau reagent, 2% Na₂CO₃, 60% Methanol

Method:

The concentration of the phenolic compounds in the extract was determined according to the method of Matthaus B. [2002]. 0.2 and 0.4mL of the above solution was added to 1mL of Folin-Ciocalteau reagent (diluted by 10 folds). 0.8mL of 2% Na₂CO₃ was added and the volume made-up to 10mL using water methanol (4:6) as the diluting fluid. The absorbance was measured at 740nm after 30 minutes using Shimadzu (1601A) UV-Vis Spectrophotometer. The concentration was calculated using gallic acid as a standard and the results was expressed as g gallic acid equivalents per 100 gram extract. The standard curve
was drawn using gallic acid as the standard (1 mg/mL.). The total phenolic content was determined as g GAE (gallic acid equivalent) using an equation from the calibration curve of gallic acid standard solution (covering the concentration range between 20 µg-100 µg).

**Determination of flavonoids**

Total flavonoid content was measured by the aluminum chloride colorimetric assay [Ebrahimzadeh M.A. *et al.*, 2008]. An aliquot of extracts (50 and 100 µg/mL) or the standard solution of quercetin (20 to 100 µg) was added to a 10 mL volumetric flask containing 4 mL of distilled H₂O. 0.3 mL 5% NaN0₂ was added. After 5 min, 0.3 mL 10% AlCl₃ was added. At 6th min, 2 mL 1 M NaOH was added and the total volume was made up to 10 mL with water. The solution was mixed well, and the absorbance was measured against prepared reagent blank at 510 nm.

**Measurement of Total antioxidant capacity using Trolox**

Trolox (6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid) is a water soluble vitamin E analog. Trolox is used to standardize antioxidants with all other antioxidants being measured in trolox equivalents.

Total antioxidant capacity of the different types of plant samples was measured by Total Antioxidant Capacity (TAC) assay kit [Bio Vision; Cat No. # K 274 – 100], which can measure either the combination of both small molecule
antioxidants or small molecule alone in the presence of protein mask [Part No. K 274 -100 - 3]. Cu\(^{2+}\) ion is converted Cu\(^{+}\) by both small molecule and protein, enabling the analysis of only small molecule antioxidants. The reduced Cu\(^{+}\) ion is chelated with a colorimetric probe giving a broad absorbance peak around 570 nm [Shimadzu UV-1700, Tokyo, Japan], proportional to total antioxidant capacity. The absorbance of the resulting oxidized solution was compared to that of calibration standard, Trolox (C\(_{14}\)H\(_{18}\)O\(_{4}\); Molar mass 250.29 g mol\(^{-1}\)). Trolox is a Hoffman – La Roche’s trade name for 6- hydroxyl – 2,5,7,8 – tetra methyl chroman – 2 – carboxylic acid, a water soluble derivative of vitamin – E.

**Reagents:**

- Cu\(^{2+}\) reagent, assay diluent, protein mask, Trolox standard (1μM).

**Method:** Samples were diluted to 1:1 with protein mask. All the well volumes were adjusted to 100mL with double distilled water. The working solution was prepared by diluting one part Cu\(^{2+}\) reagent with 49 parts of assay diluents. 100 mL of Cu\(^{2+}\) working solution added to all standard and sample well. The plate was covered and incubated at room temperature for 1 to 1.5 hours. The absorbance was measured at 570 nm using the plate reader. The results were expressed in terms of Trolox equivalent antioxidant capacity per total antioxidant unit (mM Trolox / g dry wt.) [Cook J.A. et al., 1998].
The equation to determine sample antioxidant – trolox equivalent concentration is given below:

\[
\text{Antioxidant Capacity} = \frac{\text{(Sample absorbance} - \text{blank absorbance}) \times \text{µL of sample}}{\text{Slope of standard curve}}
\]

Or,

\[
\frac{S_a}{S_v} = \frac{nM}{\mu L \text{ of } mM \text{ Trolox equivalent}}
\]

Where \( S_a \) is the sample amount (in nM) read from standard curve and \( S_v \) is the undiluted sample volume added to wells.

**Evaluation of antioxidant properties of polyphenol extracts by various *in-vitro* methods**

**1. Reducing power**

In this assay, \( \text{Fe}^{3+} \)/ ferricyanide complex is reduced to the ferrous form by antioxidants. The \( \text{Fe}^{2+} \) formed is monitored by formation of Perl’s prussian blue at 700nm. Reducing power of GLV extracts was determined according to the method of Oyaizu N, 1986.

**Chemicals:**

\( \text{NaH}_2\text{PO}_4.2\text{H}_2\text{O}, \text{Na}_2\text{HPO}_4.2\text{H}_2\text{O}, 1\% \text{Potassium Ferricyanide, 10}\%\)

\( \text{Trichloroacetic acid, 0.1}\% \text{FeCl}_3. \)
Method:

Different amounts of GLV extracts (50-250 μg) in 1mL distilled water were mixed with 2.5mL phosphate buffer (0.2M/L, pH-6.6) and 2.5mL potassium ferricyanide (1%). The mixture was incubated at 50° C for 20 minutes. 2.5mL of 10% TCA was added to the mixture. 0.5 mL of this solution was mixed with distilled water (2.5mL) and 0.5mL of 0.1% FeCl₃. The absorbance was measured at 700 nm [Shimadzu UV-1601A, Tokyo, Japan]. Reducing activity of the leafy vegetable extract was expressed as ascorbic acid equivalents from the ascorbic acid calibration curve.

Preparation of the standard curve: The standard curve was prepared with ascorbic acid (0.1mg/mL). Increased absorbance of the reaction mixture indicated increased reducing power. All analysis were done in triplicates and averaged.

2. ABTS⁺ radical cation scavenging activity.

The ABTS⁺ radical cation scavenging activity was determined according to Re R. et al., 1999.

Chemicals:

ABTS (2, 2'-azinobis- 9 (3-ethylbenzothiazoline-6-sulfonic acid), potassium per sulfate, Ethanol.
Method:

ABTS\(^{+}\) radical cation was prepared by the reaction of 5.0mL of 7mM ABTS with 88.0\(\mu\)L of 140mM potassium persulfate overnight in the dark. Prior to use in the assay, the ABTS\(^{+}\) was diluted with 50% ethanol for an initial absorbance of 0.7 at 734 nm (Shimadzu (1601A) UV-Vis Spectrophotometer) with temperature control set at 30\(^\circ\) C. Free radical scavenging activity was assayed by mixing 2.0mL diluted ABTS\(^{+}\) with 20\(\mu\)L of test antioxidant solution and monitoring the change in absorbance every minute for 5 minutes until a steady state was achieved. The antioxidant capacity of test compounds was expressed as EC\(_{50}\), the concentration necessary for 50% reduction of ABTS\(^{+}\).

3. Free radical scavenging activity using DPPH Method

Chemicals: DPPH (2, 2 diphenyl -1-picryl hydrazyl), Methanol

Method:

DPPH, a commercial oxidizing radical is reduced by antioxidants. The disappearance of DPPH radical absorption at a characteristic wavelength is monitored by decrease in optical density [Singh R.P. et al., 2002]. Different concentration of samples (20-100\(\mu\)g) was taken in different test tubes, volume adjusted to 1 mL by using distilled water. 5mL of 0.1 mM methanolic solution of DPPH was added to the test tubes and shaken vigorously. The tubes were allowed to stand at room temperature for half an hour. Control set was prepared.
as above without any extract and methanol was used for baseline correction. Changes in the absorbance of the sample were measured at 517 nm using Shimadzu (1601A) UV-Vis Spectrophotometer. Radical scavenging activity was expressed as the inhibition percentage and was calculated using the following formula.

\[
\text{Percentage radical scavenging activity} = \left( \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \right) \times 100
\]

4. Metal chelating activity:

The extracts were assessed for their ability to compete with ferrozine for iron (II) ions in free solution. Ferrous ion (Fe\(^{2+}\)) chelating by GLV extract was estimated by ferrozine assay [Dinis T.C.P. et al., 1994].

Chemicals:

FeCl\(_2\), Ferrozine (3-(2-pyridyl)-5, 6-bis (4-phenylsulfonic acid)-1, 2, 4 triazine), methanol.

Method:

GLV extract (10μg/mL) in 1mL was added to a solution of 2mM FeCl\(_2\) (50 μL). The reaction was initiated by addition of 0.2mL of 5mM Ferrozine solution. Total volume was adjusted to 4mL with methanol. The mixture was shaken vigorously and left to stand at room temperature for 10 minutes. Absorbance of the solution was then measured at 562 nm [Shimadzu UV-1700, Tokyo, Japan]
against the blank performed in the same way using FeCl$_2$ and water. Results were expressed as percentage of inhibition of the Ferrozine Fe$^{2+}$ complex formation. The percentage inhibition (% I) of the Ferrozine Fe$^{2+}$ complex formation was calculated using the formula:

$$\text{Fe}^{2+}\text{Chelating activity % I} = \frac{(\text{A}_0 - \text{A}_S)}{\text{A}_0} \times 100$$

Where A$_0$ was the absorbance of the control and A$_S$ is the absorbance of the samples. EDTA (10 µg/mL) served as the positive control, and a sample without extract and EDTA served as the negative control. All tests were run in triplicate and averaged.

5. Inhibition of lipid peroxidation in linoleic acid emulsion system:

The antioxidant activity of the GLV phenolics against lipid peroxidation was measured by the peroxidation of linoleic acid using ferric thiocyanate method (FTC) [Takao T. et al., 1994].

**Chemicals:**

Linoleic acid, Ethanol, NaH$_2$PO$_4$, 2H$_2$O, Na$_2$HPO$_4$, 2H$_2$O, Ammonium thiocyanate, Ferrous chloride, Hydrochloric acid, Ascorbic acid, α-Tocopherol, BHA.

**Method:**

Reaction mixture was prepared by using 0.2mL of GLV extract (100µg/mL)+ 0.2mL linoleic acid emulsion (25mg/mL in 99% ethanol) and 0.4 mL phosphate buffer (pH-7.4). Reaction mixture was incubated in dark at 40°C. 0.1 mL of the
reaction mixture was then added to 3 mL of ethanol (70% v/v) and 0.1 mL ammonium thiocyanate (30% w/v). After 3 minutes 20 mM/L ferrous chloride in 3.5 % (v/v) HCl (0.1ml) was added to the reaction mixture. The absorbance of the resulting solution was measured at 500nm using Shimadzu 1700 (UV-Vis) Spectrometer (Tokyo, Japan). Aliquots were assayed every 24 hours until the absorbance of the water solution (the control solution without the GLV phenolics) reached the maximum value. Butylated hydroxy anisole (BHA) and α-tocopherol was used as positive standards. The positive and negative controls were subjected to the same procedures as the samples, except that for the negative control, only the solvent was added and for the positive control, samples was replaced with α-tocopherol and BHA. All tests were run in triplicate and analysis of all samples were done in triplicate and averaged.

6. Iron mediated Fenton reaction:

A number of studies have shown that metal ions induce their toxic effects primarily through their ability to produce ROS. Iron is the most abundant transition metal in biological system. Fe^{++} has been found to react with H_{2}O_{2} to produce the extremely reactive hydroxyl radical, i.e. Fenton reaction. The radical can induce several classes of DNA damage [Jia S. et al., 2008]. Increased evidence has accumulated over the last few years on the involvement of free radical reaction in the genesis of liver cell damage. O_{2} may react with hydrogen, forming hydrogen peroxide, the generator of the powerful OH⁻ free radical in
presence of Fe^{++} and H_{2}O_{2} [Dianzani M.U. et al., 1987]. They generate some hydroxyl radicals as shown in the following reaction:

Fe^{++} + H_{2}O_{2} \rightarrow Fe^{+} + .OH + OH^{-}

Fe^{+++} + H_{2}O_{2} \rightarrow Fe^{+} + .OOH + H^{+}

Chemicals:

Iron – EDTA, 0.3 % H_{2}O_{2} (50\mu L), different concentration of GLVs (50\mu L), ethidium bromide (EtBr).

Method:

The liver tissues are homogenized using iron – EDTA. The tissues are incubated for 45 min at room temperature with 50\mu L of 0.3 % H_{2}O_{2} and 50\mu L of different concentration of GLVs in the reaction tubes. The reaction was then stopped by adding 100\mu L iron – EDTA solution. An aliquot of the reaction mixture was taken on a frosted glass slide. The slide is stained with Ethidium bromide (EtBr) and observed under florescence microscope. Different concentration of GLV extracts showed to prevent cell damage by free radicals compared with the control.

Extraction of oil:

GLVs which are mentioned in the charts were thoroughly water washed, dried and were grounded to obtain powder by laboratory grinder and sieved through
20-mesh sieve. The powders were stored in airtight containers in a refrigerator until use.

All the reagents were of analytical grade and procured from E. Merck (India) Ltd. Oil from leafy vegetable powder was extracted with n-hexane using soxhlet apparatus. After complete extraction the oil was desolventised and stored at 5-10° C for further analysis. Phospholipid content of the leafy vegetables was determined according to Official and tentative methods of American Oil Chemicals Society (AOCS), 1991.

**Determination of total phosphorous content:**

Phosphorous content was measured by the standard procedure of Chen P.S. and Toribara T.Y, 1951.

**Preparation of reagents:**

Standard solution of potassium dihydrogenphosphate (KH$_2$PO$_4$) was prepared by dissolving 15 mg anhydrous KH$_2$PO$_4$ in 100 mL of glass distilled water. 2.5 g of ammonium molybdate was dissolved in hot glass distilled water. 10 mg of dry and fresh ascorbic acid was dissolved in 100 mL glass distilled water. 6(N) sulphuric acid was prepared from 36 (N) sulphuric acid (BDH, A.R. quality). Colour reagent was prepared by mixing 1 volume of 2.5% ammonium molybdate, 1 volume of 10% ascorbic acid, 1 volume of 6(N) sulphuric acid and 2 volume of glass distilled water.
Procedure:

Preparation of standard curve

A standard curve was prepared with known concentration (3 to 8 µg of phosphorous) of standard phosphate solution against their absorbance at 820 nm. 0.1 mL, 0.15 mL and 0.2 mL of standard phosphate solution were taken in a test tube in triplicate set and volume of the solution was made upto 4 mL with glass-distilled water. 4 mL of colour reagent was added to each set. The solution was mixed properly and placed at 37°C water bath for 90 minutes. After cooling at room temperature, absorbance was recorded at 820 nm against the reagent blank in a UV-Vis Spectrophotometer (Shimadzu UV Vis 1601, Tokyo, Japan).

For the estimation of phosphorous in sample, 1 mL of diluted sample was taken in test tube and 0.4 mL of perchloric acid (72%, BDH, A.R. quality) was added to each test tube and was heated below 180°C until the solution became clear. After cooling the volume was made to 4 mL with distilled water and 4 mL of colour reagent was added and the same method was followed as described for preparation of standard curve. The phosphorous content was multiplied by factor 25 to obtain the total phospholipids content.

Fatty Acid composition

The equipment used in this analysis was an analytical gas chromatograph (Agilent 6890 Series Gas Chromatograph) equipped with a flame ionization detector (FID). The DB-WAX capillary column (J&W Scientific Columns from...
Agilent Technologies, USA) employed for the analysis was 30 meter (length) × 0.25 mm (i.d), 0.25 mm (film thickness). Two ANALAB GC Generator; HG 300 (Hydrogen generator) and N-A-300 (air and nitrogen generator) was employed for the GC gas supply. The GC inlet temperature and FID detector temperature was maintained at 250°C. 30 mL/min hydrogen flow, 300 mL/min air flow and 29 mL/min nitrogen flow was maintained in the FID detector. 1 mL/min carrier gas (Nitrogen) flow was maintained in the capillary column. Oven temperature was maintained at 150°C for 2 minutes, then 15°C/minutes upto 190°C, then 5 minutes hold, 4°C/minutes upto 230°C, 10 minutes hold. The sample was dissolved in hexane (10 mg/mL) and 1 μL of the sample was injected from Agilent D08-B1940 microsyringe (1-10 μL) and the respective chromatogram was obtained. The peaks were identified by comparing the retention time of the peaks with those of standard samples separated on the same column under same conditions. The percentage of each component was calculated by the software provided by the Agilent Technologies Chemstation Family, based on the method of dividing the area of each peak by the total area of the peaks. Standard methyl esters were purchased from Sigma Chemical Co., St. Louis, MO, USA.

**Processing Polyphenols**

GLVs are generally consumed in the cooked form apart from salads. Therefore there is a need to assess the changes that occur in the antioxidant activity on cooking.
Preparation of samples:

GLVs were washed with tap water after removing manually the inedible parts using a sharp knife. They were dried on paper towels and were cut into almost equal pieces. All the LVs were divided into four portions (10 g for each application). One portion was retained raw; others were cooked in three different methods in triplicate as given below.

Cooking conditions were determined with a preliminary experiment for each vegetable [Jimenez-Monreal A.M. et al., 2009].

Boiling:

GLVs (10 g) were added to 100 mL water which has just reached to boil in a stainless steel pan and cooked until tender. The water was evaporated. The samples were cooled rapidly on plenty of ice.

Frying:

GLVs (10 g) were added to 50 mL mustard oil in a wok and fried (180°C) for 5 minutes until the sample becomes crisp tender. The fried samples dabbed with blotting paper to allow absorption of excess oil. Samples were cooled rapidly for the antioxidant assays.

Microwave cooking:

GLVs (10 g) was placed in a glass container and 5 mL distilled water was added. Then the samples were cooked in the domestic microwave oven (LG
Electronics India Ltd. Model: MS 257 PL Korea) for 5 minutes. Samples were drained off and cooled rapidly in ice.

The raw and processed vegetables were homogenized in a homogenizer for 5 minutes. The homogenized samples were kept at 4 °C until further analysis.

**Determination of Total Phenolic Content**

The concentration of the phenolic compounds in the extract was determined according to the method of Matthaus B. [2002] as mentioned above.

**Determination of Total Antioxidant activity**

Antioxidant activity was determined by the DPPH Method of Zhang D. and Hamazu Y. [2004] with some modification.

Vegetable content of the methanol extracts of fresh and cooked vegetables were adjusted to 6μg/mL, which was chosen as appropriate concentration for assessing the antioxidant activity after preliminary studies with different concentrations. An aliquot of 5 mL of 0.1mM DPPH radical in methanol was added to test solution with 0.5mL of vegetable extract (at 6μg/mL). Pure methanol was used as the control. The reaction mixture was vortex mixed and let to stand at room temperature in the dark for sixty minutes, before the decrease in absorbance at 517nm was measured. Pure methanol was used to calibrate the spectrophotometer. Antioxidant activity was expressed as percentage inhibition of DPPH radical and is expressed as percentage radical scavenging activity.
% radical scavenging activity = \frac{(\text{Control OD} - \text{Sample OD})}{\text{Control OD}} \times 100

Fourier Transform Infra - Red Spectrophotometer (FTIR) analysis of polyphenols

The extracts of polyphenols were concentrated to a dry mass by distillation under vacuum and were analyzed by standard procedure through KB pellet preparation in a Fourier Transform Infra – Red Spectrophotometer instrument [More A.B. et al., 2005] (Spectrum One Version A; Perkin Elmer, USA; Serial No: 57937). KB pellet preparation: about 1 to 2 mg of the previously dried sample and 100 mg to 200 mg of KBr previously dried at 155 ± 5°C for 5 hours mixed intimately in a suitable electrically operated small ball mill and pressed in a suitable dye under high pressure (at least 25000 psi) into a small disc that measured about 10 mm in diameter and about 1 mm thickness. Ideally, a clear and transparent pellet is obtained which actually consists of solid solution of the substance in KBr.

Separation of Polyphenols by HPLC

Extraction and Hydrolysis:

The extraction method employed for the GLV extracts was the method of Hertog M.G.L et al., [1993].
Chemicals:

Aqueous methanol, BHA [2(3)-tert-butyl-4-hydroxy anisole], 6M HCl

Method:

The freeze dried samples of the fresh leaves (0.5g) were weighed into 100mL Erlenmeyer flask. It is then dispersed into 40mL 62.5% aqueous methanol containing 2g/L BHA. Mixture was ultrasonicated for 5 minutes. To this extract was added 1mL of 6M HCl with careful mixing. The extraction solution thus obtained consisted of 1.2M HCl in 50% aqueous methanol. The sample was bubbled with nitrogen for 40-60 seconds, after which the flask was sealed tightly.

Hydrolysis was carried out in a shaking water bath at 90°C for 2 hours. After hydrolysis, the extract was allowed to cool and filtered and subsequently made upto 100mL with methanol and sonicated for 5 minutes. Approximately 2mL was filtered through a 0.2μm membrane filter prior to injection in HPLC.

The components were then separated by HPLC according to the method of Siddhuraju P. and Becker K. [2003]. Separation was done in a 4.6x250mm ultrasphere C18 reversed phase column. Gradient elution was employed for flavonoids with a mobile phase consisting of 50mM H3PO4, pH-2.5 (Solution A), and acetonitrile (Solution B) as follows: isocratic elution 95% A/5% B, 0-5 minutes; linear gradient from 95% A/5% B to 50% A/50% B, 5-55 minutes;
Isocratic elution 50% A/50% B, 55-65 minutes; linear gradient from 50% A/50% B to 95% A/5% B, 65-67 minutes; post time 6 minutes before next injection.

The system, WATERS 2487 is equipped with a C-18 column (Nova-Pak C\textsubscript{18}, 3.9x150mm), 280, 340 and 370nm wavelengths were selected for the detection. The flow rate of the mobile phase was 0.8 mL/min and the injection volume was 20\textmu L of the standards and sample extracts. The peaks were identified in comparison with authentic standards.

**Statistical analysis:**

The data was expressed as mean ± SEM. Differences among the experimental groups were analyzed using one way ANOVA and the comparisons between the means were carried out using the Tukey test; \( p<0.05 \) was considered as statistically significant in all the experiments.