

## COMPARATIVE ANTIOXIDANT STUDIES OF ETHANOL EXTRACT AND FRESH AQUEOUS EXTRACT OF *VETIVERIA ZIZANIOIDES*

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### ABSTRACT

Primary sources of naturally occurring antioxidants are whole grains, fruits and vegetables. Most of the antioxidant compounds in a typical diet are derived from plant sources and belong to various classes of compounds with a wide variety of physical and chemical properties. Oxidative damage plays a significantly pathological role in human disease. Free radicals lead to cellular necrosis, which is implicated in some membrane pathophysiological conditions including atherosclerosis, rheumatoid arthritis as well as toxicity of many xenobiotic, ischemia and injury of many tissues, central nervous system injury, gastritis, ageing, inflammatory response syndrome, respiratory diseases, liver diseases, and cancer. Many herbal plants contain antioxidant compounds and these compounds protect cells against the damaging effects of reactive oxygen species (ROS), such as singlet oxygen, superoxide, hydroxyl radicals and peroxy nitrite. The plant *Vetiveria Zizanioides* was selected based on Ayurvedic literature claim many medicinal uses. So the plant was subjected to antioxidant studies. Result shows better activity in case of aqueous extract prepared from fresh plants compared to ethanolic extract of dried plant.

**Keywords:** Antioxidant, Aqueous Fresh extract, Ethanolic extract, DPPH, Scavenging.

### INTRODUCTION

*Vetiveria zizanioides* is popularly known as Khas Khas, Khas or Khus grass in India. *Vetiveria zizanioides*, a member of the family Poaceae. Commonly known in other Indian languages Marathi Vala. Telugu Kuruveeru, Vettiveellu, Vettiveerum, Tamil Vattiver, Kannad Vattiveeru, Laamancha, Kaddu, Malayalam Ramaccham, Vettiveru<sup>1</sup>

*Vetiveria zizanioides* (Fig : A<sub>1</sub>) is a densely tufted grass with the culms arising from an aromatic rhizome up to 2m tall; the roots are stout, dense and aromatic; leaves are narrow, erect, keeled with scabrid margins; inflorescence is a panicle, up to 15-45 cm long of numerous slender racemes in whorls on a central axis; 440 spikelet's are grey to purplish, 4-6 mm long, in pairs, one sessile the other pedicelled; 2-flowered; the lower floret is reduced to a lemma, upper bisexual in sessile, male in the pedicelled spikelet; glumes are armed with stout, tubercle-based spines<sup>2</sup>



Fig. A<sub>1</sub>: *Vetiveria zizanioides*

It is found throughout the plains and lower hills of India, particularly on the river banks and in rich marshy soil. *Vetiver* has been known to India since ancient times. It has been considered as a high class perfume and copper plate inscriptions list the perfume as one of the articles used by royalty. Two species of *Vetiveria* are found in India, of which *Vetiveria zizanioides* is the common source of the well known oil of *vetiver*, which is used in medicine and in perfumery<sup>3</sup>.

*Vetiveria zizanioides* is profoundly used in Ayurvedic medicine. The chemical constituents present in the plant are Vetiverol, Vetivone, Khusimone, Khusimol, Vetivene, Khositone, Terpenes, Benzoic acid, Tripene-4-ol,  $\beta$ -Humulene, Epizizianal, vetivenyl vetivenate, iso khusimol,  $\beta$ -vetivone, vetivazulene. Ayurvedic literature mentioned that plant is used as digestive, carminative stomachic, constipating, haematinic, expectorant, antispasmodic, antiasthmatic, antigitout, anthelmintic, antimicrobial and diuretic<sup>4</sup>.

The root (Fig A<sub>2</sub>) are used for cooling to the brain, and also used in treatment of ulcers. In addition to these, the plant is used for anaemia, amenorrhoea and dysmenorrhoea. *Vetiver* oil possesses sedative property and has been traditionally used in aromatherapy for relieving stress, anxiety, nervous tension and insomnia<sup>5</sup>.



Fig. A<sub>2</sub>: *Vetiveria zizanioides* roots

We decide to study by comparison of In-vitro antioxidant studies which we carried out on whole plant of *Vetiveria zizanioides*, in both aqueous extract of fresh and ethanolic extract of dried plant. To establish that in which form of plant is most suitable of best use.

### MATERIAL AND METHODS

#### Chemicals and Reagents

Chemicals used in our study were ascorbic acid, ethylenediamine tetraacetic acid, ferrous ammonium sulphate, Phosphomolybdic

acid, sodium tungstate, potassium ferricyanide and sodium nitroprusside, trichloroacetic acid, naphthylethylenediamine dihydrochloride, sodium nitrite, phosphoric acid, nitro bluetetrazolium, phenazine methosulfate are obtained from SD Fine Chemicals Ltd, India. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) obtained from Sigma-Aldrich, U.S.A. All other reagents and solvents used in the study were of analytical grade.

#### Plant Material

Whole Plant of *Vetiveria zizanioides* was collected with roots in the month of August from Salem district, Tamil Nadu, India. The plant material was authenticated at the Department of Botany, Karpagam University, Coimbatore and Tamilnadu. A voucher specimen as a herbarium (16/09KU/2009) has been kept in museum for future reference. The plants were chopped and dried at room temperature for 10 days and used as raw material. The dried plants were powdered using mechanical method and resulting powder was passed through the 40 # sieve and stored in the airtight container.

#### Preparation of crude aqueous extract

Fresh plants were chopped in small pieces and then weighed accurately 250 gm transferred in a stainless steel vessel and mixed with 2litre of distilled water. Then the mixture was boiled for about 2 hours, and then mixture was filtered by using vacuum filter assembly. Then the filtrate was evaporated on hot plate until it reaches the concentrated quantity.

#### Preparation of ethanolic extracts

Then weighed accurately 250 g of dried powder of drug was packed in thimble flask and 750ml of ethanol (70%) was added in 1 litre round bottom flask. Then the Soxhlet assembly was set up to complete 10 to 15 cycles. After that the extract was filtered and filtrate was concentrated using water bath. The obtained extract was kept in a desiccator for 3 days, to carry out the antioxidant studies.

#### Preliminary Phytochemical Screening

The Aqueous and alcoholic extract were taken for various qualitative chemical tests to determine the presence of various phytoconstituents like alkaloids<sup>6</sup>, glycosides<sup>7</sup>, carbohydrates<sup>8</sup>, phenolic and tannins<sup>9</sup>, phytosterols<sup>10</sup>, fixed oils, protein and amino acids, flavanoids, saponins, gums and mucilage<sup>11</sup> using reported method. The aqueous extract shows the presence of glycosides<sup>7</sup>, carbohydrates<sup>8</sup>, phenols, flavanoids, saponins, gums and mucilage<sup>11</sup>

#### Estimation of Total Phenolic Content by Spectrophotometer

By Folin – Denis Method The method is based on the oxidation of molecule containing a -OH groups. The tannin and tannin like compound reduce Phosphotungstomolybdic acid in alkaline solution to produce a highly blue colour solution<sup>12, 13, 14</sup>. 1ml of the aqueous and ethanolic extract that has adjusted to come under the linearity range i.e. (50µg/ml) of both the extract was withdrawn in 10ml volumetric flask separately. To each flask 0.5ml of Folin-Denis reagent and 1ml of Sodium carbonate was added and volume is made up to 10ml with distilled water. The absorbance was measured at absorption maxima 700nm within 30 minute of reaction against the blank. The total phenolic content was determined by using calibration curve (5 to 30µg/ml). Three readings were taken for each and every solution for checking the reproducibility and to get accurate result. Results are provided in (Table 1 and Figure 1). The intensity of the solution is proportional to the amount of tannins and can be estimated against standard tannic acid, the total phenolic content, expressed as mg tannic acid equivalents per 100 g dry weight of sample.

#### Total Flavonoid Content by Spectrophotometer

##### Aluminium chloride colorimetric assay method

Total flavonoid contents were measured with the aluminium chloride colorimetric assay<sup>15</sup>. Aqueous and ethanolic extracts that has been adjusted to come under the linearity range i.e. (400µg/ml) and different dilution of standard solution of Quercetin (10-100µg/ml) were added to 10ml volumetric flask containing 4ml of water. To the above mixture, 0.3ml of 5% NaNO<sub>2</sub> was added. After 5

minutes, 0.3ml of 10% AlCl<sub>3</sub> was added. After 6 min, 2ml of 1 M NaOH was added and the total volume was made up to 10 ml with distilled water. Then the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. Results are tabled in (Table 2 and Figure 2). Total flavonoid content of the extracts was expressed as percentage of Quercetin equivalent per 100 g dry weight of sample.

#### In-Vitro Antioxidant Study

##### FRAP method<sup>16</sup>

The ferric reducing property of the extract was determined by taking 1ml of different dilutions of standard solutions of Gallic acid (10 -100 µg/ml) or aqueous and ethanolic extract that has adjusted to come under the linearity range (500µg/ml) was taken in 10ml volumetric flasks and mixed with 2.5ml of potassium buffer (0.2 M, pH 6.6) and 2.5ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20m in. Then 2.5ml of 10% trichloroacetic acid was added to the mixture to stop the reaction. To the 2.5ml of above solution 2.5ml of distilled water is added and then 0.5ml of 0.1% of FeCl<sub>3</sub> was added and allowed to stand for 30 min before measuring the absorbance at 593 nm. Results are provided in (Table 3 and Figure 3). The absorbance obtained was converted to Gallic acid equivalent in mg per gm. of dry material (GAE/g) using Gallic acid standard curve.

#### Scavenging Activity Assays

##### Nitric oxide scavenging assay

Nitric oxide radical inhibition was estimated by the use of Griess Illosvory reaction<sup>17,18</sup>. In this investigation, Griess Illosvory reagent was generally modified by using Naphthyl ethylene diamine dihydrochloride (0.1%w/v) instead of the use of 1-naphthylamine (5%). The reaction mixture (3ml) containing 2ml of 10 mM sodium nitroprusside, 0.5ml saline phosphate buffer and 0.5ml of standard solution or aqueous and ethanolic extract of (50 -500µg/ml) were incubated at 25°C for 150min. After incubation, 0.5ml of the reaction mixture was mixed with 1ml Sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5min for the completion of the reaction of diazotization. After that further 1ml of the Naphthyl ethylene diamine dihydrochloride was added, mixed and was allowed to stand for 30min at 25°C. The concentration of nitrite was assayed at 546nm and was calculated with the control absorbance of the standard nitrite solution (without extracts or standards, but the same condition should be followed). Here the blank is taken as the buffer and make up solvents and the Ascorbic acid (10 -50 µg/ml) was taken as standard. Results are represented in (Figure 4,5 ). The percentage inhibition was calculated using the formula:

$$\% \text{ Scavenging Activity} = \frac{\text{A control} - \text{A test or A Std}}{\text{A control}} * 100$$

Where, A control = absorbance of control

A test or A Std = absorbance of test or std

##### Hydrogen Peroxide Scavenging Assay<sup>19</sup>

The ability of extracts to scavenge hydrogen peroxide was determined by little modification here the solution of hydrogen peroxide (100mM) was prepared instead of 40mM in phosphate buffer saline of (PH 7.4), at various concentration of aqueous and ethanolic extract (50 -500 µg/ml) were added to hydrogen peroxide solution (2 ml). Absorbance of hydrogen peroxide at 230 nm was determined after 10 minutes against a blank solution containing phosphate buffer without hydrogen peroxide. For each concentration, a separate blank sample was used for back ground subtraction. In case of control takes absorbance of hydrogen peroxide at 230 nm without sample extracts. Results are provided in (Figure 6,7). The percentage inhibition activity was calculated from  $[(A_0 - A_1)/A_0] \times 100$ , where A<sub>0</sub> is the absorbance of the control and A<sub>1</sub> is the absorbance of extract/standard taken as Gallic acid (10 -100 µg/ml).

##### DPPH –RSA method

The free radical scavenging activity of aqueous and ethanolic extracts and the standard L-Ascorbic Acid (Vitamin C) was measured

in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH<sup>20,21</sup>. Here, 0.1mM solution of DPPH in alcohol was prepared and it must be protected from light influence by maintaining the dark condition and also fold by aluminium foil and 3ml of this solution was added to 1ml various conc.(100-2000 µg/ml) of extracts or standard solution of (10-100 µg/ml). Absorbance was taken after 30min at 517nm. Results are provided in (Figure 8-9). The percentage inhibition activity was calculated from  $[(A0-A1)/A0] \times 100$ , where A0 is the absorbance of the control and A1 is the absorbance of extract/standard taken as Ascorbic acid.

**RESULTS AND DISCUSSION**

**Effect of TPC & Flavonoid Content**

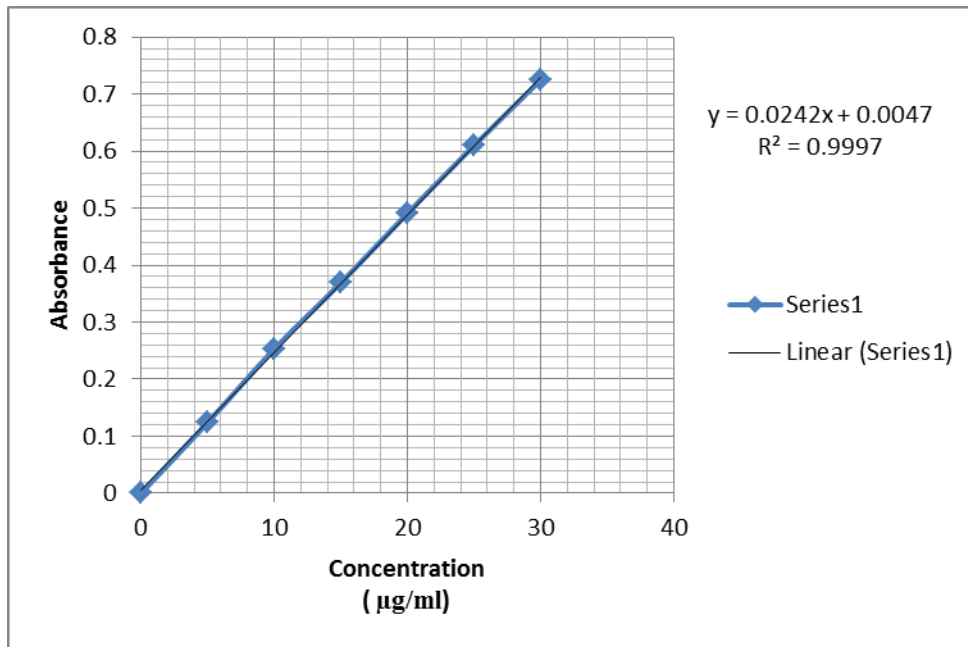
The quantitative determination of the total phenolic content, expressed as mg tannic acid equivalents and per 100 g dry weight of sample TPC of L.S ethanolic and aqueous extracts showed the

content values of 18.45±0.21%w/w and 15.43±0.31%w/w (Table 1 and Figure 1) (Table 2 and Figure 2) and total flavonoid content of the extracts was expressed as percentage of Quercetin equivalent per 100 g dry weight of sample the total flavonoids estimation of aqueous and ethanolic extracts showed the content values of 0.94±0.31%w/w and 1.21±0.21%w/w. The above results showed that aqueous contain more tannins and flavonoid content than the alcoholic extract. It may due to the solubility of principle contents presence be higher in case of aqueous solvent because of higher polarity in comparison with alcohol.

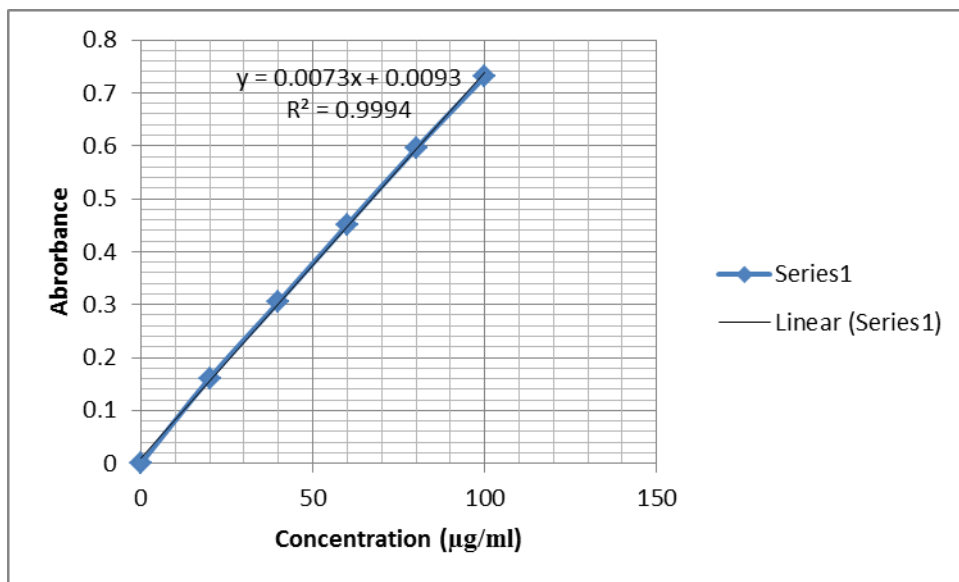
**Table 1 : Total phenolic content**

S.No	Concentration of extract	% w/w of total tannin
1	Aqueous extract 50µg/ml	15.43±0.21
2	Ethanol extract 50µg/ml	18.45±0.31

Values are mean ±S.E.M, n=3



**Fig. 1: Total Phenolic Content (Tannic acid ) Vetiveria zizanioides**



**Fig. 2: Total Flavonoid Content Vetiveria zizanioides Quercetin std.**

Table 2: Total Flavonoid Content

S. No	Concentration of extract	% w/w of total Flavonoid (Quercetin)
1	Aqueous extract 50µg/ml	0.94±0.21
2	Ethanol extract 50µg/ml	1.21±0.31

Values are mean ±S.E.M,n=3

#### Capacity of FRAP Method

At low pH, measuring the change in absorption at 593 nm can monitor reduction of a ferric complex to the ferrous form, which has an intense bluish green color. The change in absorbance is directly

related to the combined or "total" reducing power of the electron-donating antioxidants present in the reaction mixture. Here the FRAP showed the results of aqueous and ethanolic extracts that of 51.47±0.43mg equivalent to Gallic acid(GAE)/g of sample and 59.78±0.26mg GAE/g (Table 3 and Figure 3) of sample respectively.

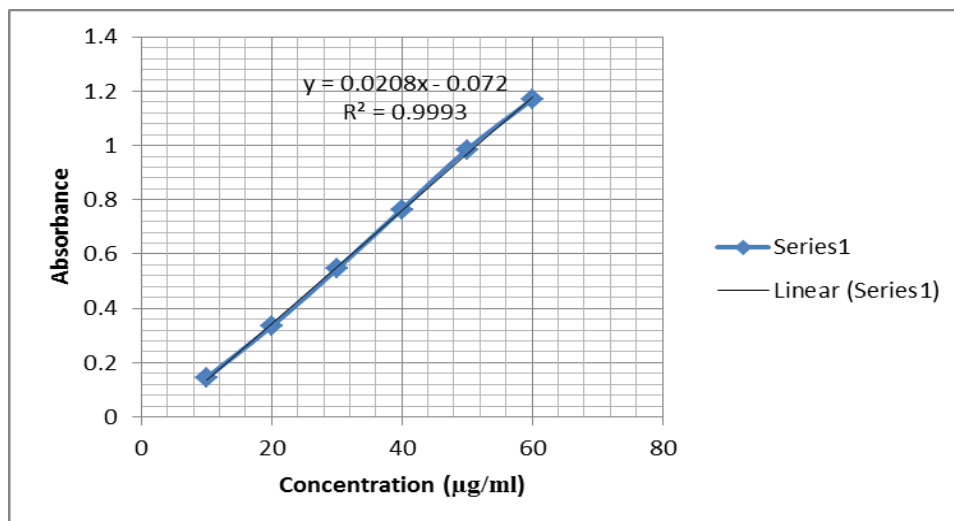
Fig. 3: FRAP Method: *Vetiveria zizanioides* ; Gallic Acid Std

Table 3:

S.No	Concentration of extract	Mg GAE/g of extract
1	Aqueous extract 500µg/ml	51.47±0.43
2	Ethanol extract 500µg/ml	59.78±0.26

Values are ± S.E.M, n=3

#### Capacity of Nitric Oxide Scavenging Assay

Nitric oxide is a very unstable species under the aerobic condition. It reacts with O<sub>2</sub> to produce the stable product nitrates and nitrite through intermediates through NO<sub>2</sub>, N<sub>2</sub>O<sub>4</sub> and N<sub>3</sub>O<sub>4</sub>. It is estimated by using the Griess reagent. In the presence of test compound, which is a scavenger, the amount of nitrous acid will decrease. The extent

of decrease will reflect the extent of scavenging, the %inhibition of aqueous and ethanolic extract of three parallel readings of (r<sub>2</sub>=0.9996) and (r<sub>2</sub>=0.9995). Aqueous and ethanolic extract showed that I<sub>c</sub> 50 values 258.00µg/ml and 235.06µg/ml (Figure 5) respectively as compared to the standard of Ascorbic acid of 34.16µg/ml (r<sub>2</sub>=0.9999) (Figure 4).

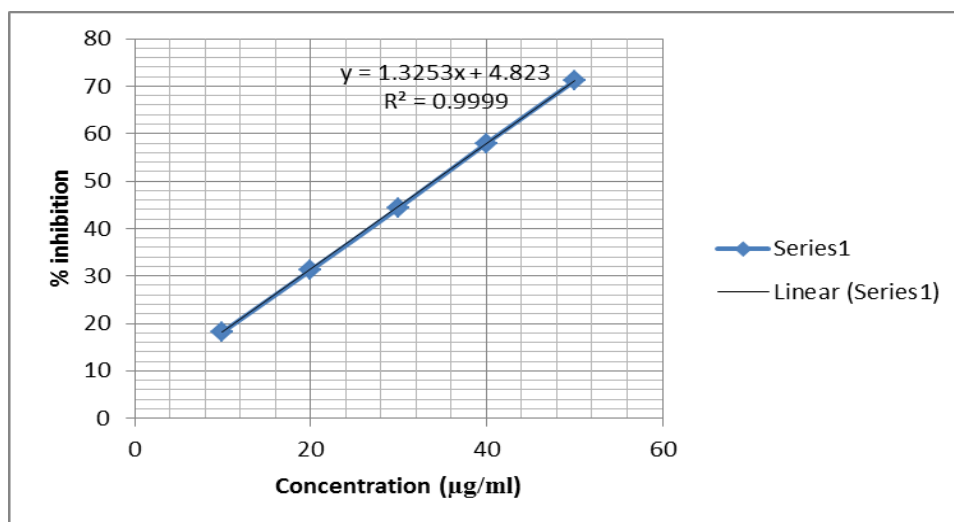


Fig. 4: Nitric oxide radical scavenging assay , Ascorbic acid STD

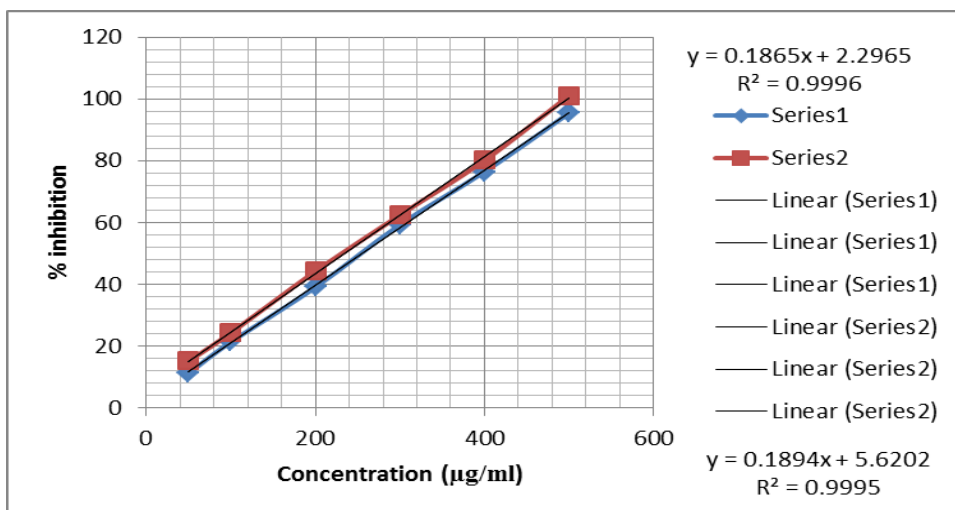


Fig. 5: Nitric oxide radical scavenging assay *Vetiveria zizanioides*.

Series 1 shows Aqueous extract Series 2 shows Ethanollic extract

**Capacity of Hydrogen Peroxide Scavenging**

H<sub>2</sub>O<sub>2</sub> itself is not very reactive, but it can sometimes be toxic to the cell because it may give rise to hydroxyl radical in the cells. Thus, removal of H<sub>2</sub>O<sub>2</sub> is very important for protection of food systems.

Scavenging of Hydrogen per oxide and its %inhibition of aqueous and ethanollic extract showed that I<sub>c</sub> 50 values 245.60µg/ml (r<sub>2</sub>=0.9991) and 234.21µg/ml (r<sub>2</sub>=0.9997) (Figure 7) respectively. Gallic acid has taken as reference which showed 58.00µg/ml. (r<sub>2</sub>=0.9997) (Figure 6)

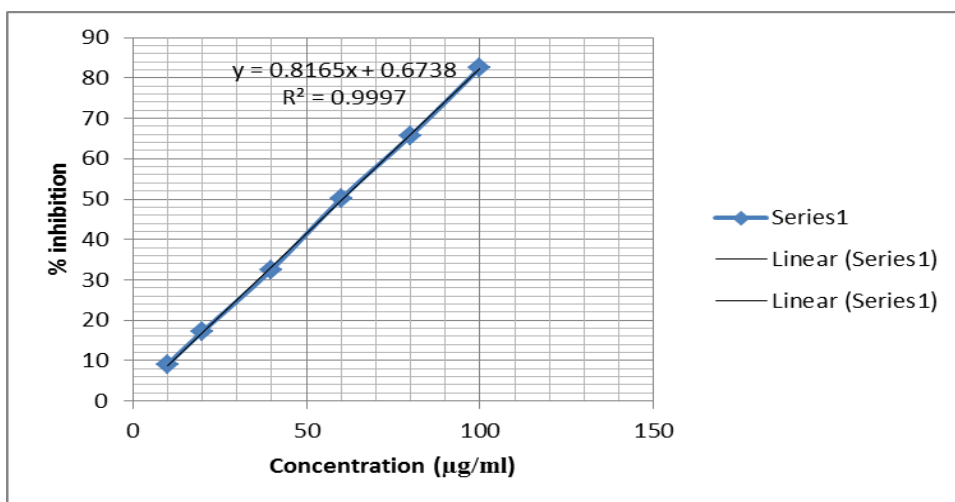


Fig. 6: Hydrogen Peroxide Scavenging Assay *Vetiveria zizanioides* STD Gallic acid

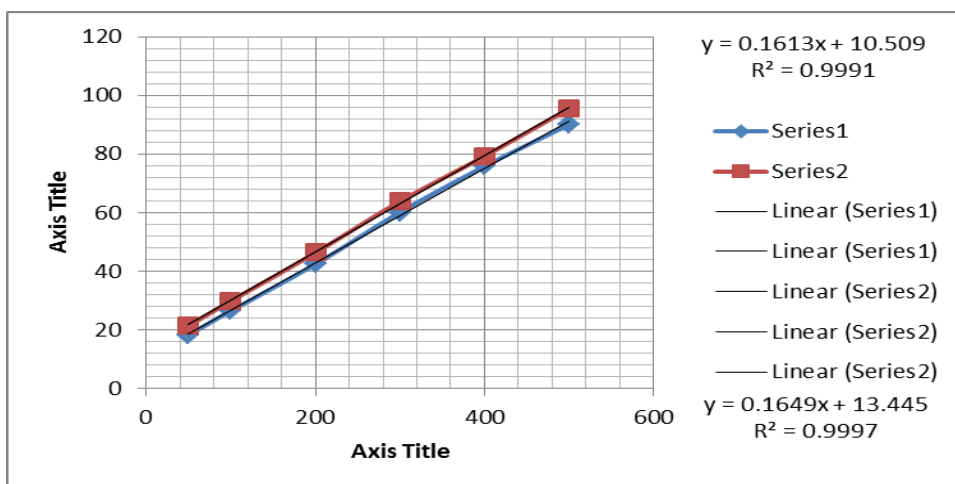


Fig. 7: Hydrogen Peroxide Scavenging Assay *Vetiveria zizanioides*.

Series 1 Aqueous extract

Series 2 ethanolic extract

#### Capacity of DPPH –RSA

The antioxidant reacts with stable free radical, DPPH and converts it to 1, 1- Diphenyl-2- Picryl Hydrazine. The ability to scavenge the free

radical, DPPH was measured at an absorbance of 517 nm. So the DPPH –RSA and its % inhibition of aqueous and ethanolic extract showed that IC50 values 241.24 µg/ml ( $r_2=0.9992$ ) and 215.11µg/ml ( $r_2=0.9998$ ) (Figure 9) respectively. Ascorbic acid has taken as reference which showed 52.12 µg/ml. ( $r_2=0.9998$ ) (Figure 8) among these results fresh aqueous extract has more potent than dried plants alcoholic extract.

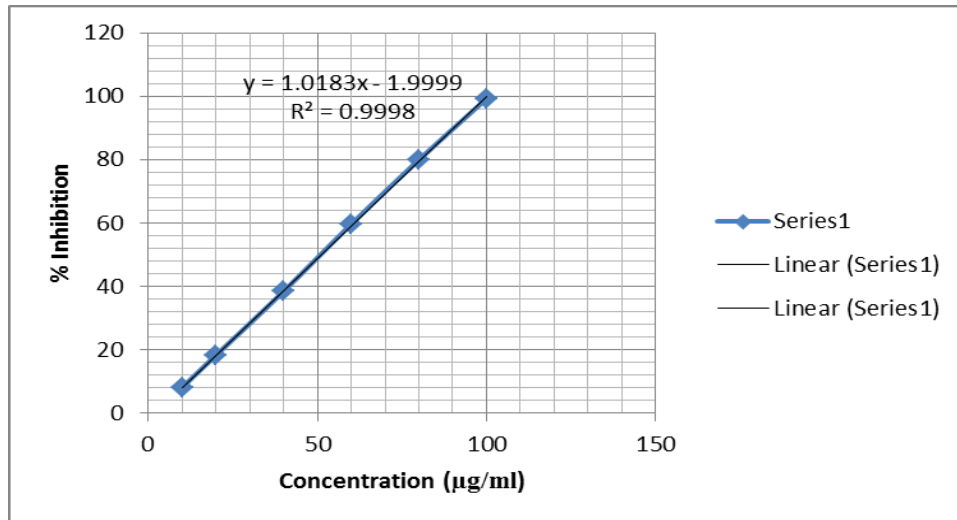


Fig. 8: DPPH Radical Scavenging assay *Vetiveria zizanioides* STD Ascorbic acid

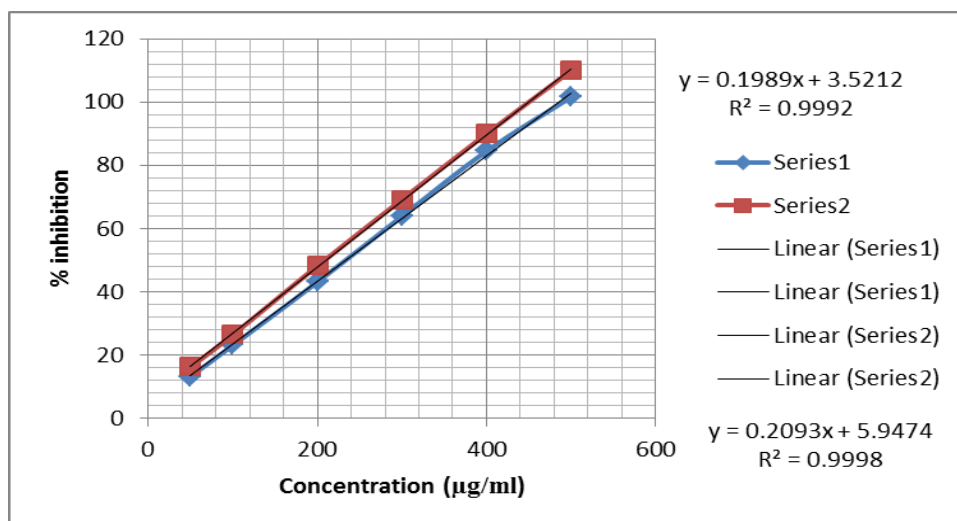


Fig. 9: DPPH Radical Scavenging assay *Vetiveria zizanioides*.

Series 1 Aqueous extract; Series 2 Ethanolic extract

#### CONCLUSION

It can be concluded that *Vetiveria zizanioides* plant possesses the antioxidant substance which may be potential responsible for the treatment of various diseases. But it is evident from the above study that fresh plant alcoholic extracts are more potent antioxidant in comparison of aqueous extract.

The dried plant extract contain more antioxidant substance which are highly soluble in alcohol. The total flavonoids content in ethanolic extract is more than aqueous extract flavonoids are well known for their antioxidant properties. More over phenolic contents of ethanol extract is also more than aqueous extract which again are well established antioxidant substances. So it is evident that fresh plant aqueous extract do not show better activity. Our further investigation is going on to isolate the responsible component in alcohol extract due which the extract showing very good antioxidant properties.

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