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RESEARCH ARTICLE

Evaluation of *In Vitro* Antioxidant Activity of Ethanolic Extract from *Vallaris solanacea* Leave

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Abstract

Several methods employed in evaluating antioxidant activities on varied samples gives varying results depending on the specificity of the free radical or oxidant used as a reactant. This study investigated the anti-oxidation /radical scavenging properties since ethanolic extraction on *Vallaris solanacea* leaves. Antioxidant capacity on *Vallaris solanacea* leaves were evaluate near measuring a DPPH photometric assay, Superoxide radical scavenging activities, Iron chelating activities, Hydroxyl radical scavenging activity, Nitric oxide radical scavenging activities, Total antioxidant activities, Ferric Reducing Ability Power (FRAP) assay, full phenol content overall flavonoids content. A concentration was 125, 250, 500, 1000 µg/ml. The standard was used according to their methods. All values are expressed for mean ± SEM for three determinations with percentage of activity (±SEM) of IC50 values. The result revealed that DPPH as 625µg/ml, Superoxide radical scavenging as 459µg/ml, Iron chelating activity as 639µg/ml, Hydroxyl radical scavenging as 556µg/ml, Nitric oxide original scavenging as 683µg/ml, Total antioxidant as 638 µg/ml, FRAP as 543µg/ml and the total phenolic , flavonoids content was 3.980±0.007 and 2.651±0.077. The findings from the study reveal that the antioxidative potentials of *Vallaris solanacea* leaves of ethanolic extract and could serve as free radical inhibitors, phenolic and flavonoids as possibly acting as principal antioxidants

Keywords: *Vallaris solanacea antioxidant, Phenols, Flavonoids, Free radical.*

Introduction

Materials and Methods

Collection and Identification of *Vallaris solanacea*

The leaves parts of *Vallaris solanacea* were collected from kanchipuram, Thirunelveli District of Tamil Nadu, India. Taxonomic identification was made from Botanical Survey of Medicinal Plants Unit Siddha, Government of India. Palayamkottai. The leaves parts of *Vallaris solanacea* were dried under shade, segregated, pulverized by a mechanical grinder and passed through a 40-mesh sieve. The powdered plant materials were stored in an airtight container.

Preparation of Extracts from *Vallaris solanacea*

The above powdered materials were successively extracted with ethanol by hot continuous percolation method in Soxhlet apparatus for 24 hrs. The extract was concentrated by using a rotary evaporator and subjected to freeze drying in a lyophilizer till dry powder was obtained.

DPPH Photometric Assay [1]

A methanol solution of 0.5ml of DPPH (0.4mM) was added to 1ml of the different concentrations of plant extract and allowed to react at room temperature for 30 minutes. Methanol served as the blank and DPPH in methanol without the extracts served as the positive control. After 30 min, the absorbance was measured at 518nm and converted into percentage radical scavenging activity as follows.

$$Scavengingactivity\,(\%) = \frac{A_{518}\,\text{Control} - A_{518}\,\text{Sample}}{A_{518}\,\text{Control}} \times 100$$

Where A_{518} control is the absorbance of DPPH radical with methanol; A_{518} sample is the absorbance of DPPH radical with sample extract/ standard.

Reagents

- 0.4mM, DPPH.

Superoxide Radical Scavenging Activity [2]

Superoxide radical (O_2^-) was generated from the photo reduction of riboflavin and was detected by nitro blue tetrazolium dye (NBT) reduction method. The assay mixture contained sample with 0.1ml of Nitro blue tetrazolium (1.5 mM NBT) solution, 0.2 ml of EDTA (0.1M EDTA), 0.05 ml riboflavin (0.12 mM) and 2.55 ml of phosphate buffer (0.067 M phosphate buffer). The control tubes were also set up where in DMSO was added instead of sample. The reaction mixture was illuminated for 30 min and the absorbance at 560 nm was measured against the control samples. Ascorbate was used as the reference compound. All the tests were performed in triplicate and the results averaged. The percentage inhibition was calculated by comparing the results of control and test samples.

Reagents

- 1.5mM, NBT.
- 0.1M, EDTA.
- 0.12mM, Riboflavin.
- 0.067M, Phosphate buffer.

Iron Chelating Activity [3]

The method of Benzie and strain (1996) was adopted for the assay. The principle is based on the formation of *O*-Phenanthroline-Fe^{2+} complex and its disruption in the presence of chelating agents. The reaction mixture containing 1 ml of 0.05% O-Phenanthroline in methanol, 2 ml ferric chloride (200µM) and 2 ml of various concentrations ranging from 10 to 1000µg was incubated at room temperature for 10 min and the absorbance of the same was measured at 510 nm. EDTA was used as a classical metal chelator. The experiment was performed in triplicates.

Reagents

- O-Phenanthroline (0.05%).
- 200µM, Ferric chloride.

Hydroxyl Radical Scavenging Activity [4]

The assay is based on quantification of degradation product of 2-deoxy ribose by condensation with TBA. Hydroxyl radical was generated by the Fe^{3+}-Ascorbate-EDTA-H_2O_2 system (Fenton reaction). The reaction mixture contained 0.1 ml deoxyribose (2.8mM),0.1 ml EDTA (0.1 mM), 0.1 ml H_2O_2 (1mM), 0.1 ml Ascorbate (0.1mM), 0.1 ml KH_2PO_4-KOH buffer, P^H 7.4 (20mM) and various concentrations of plant extract in a final volume of 1 ml. The reaction mixture was incubated for 1 hour at 37°C. Deoxyribose degradation was measured as TBARS and the percentage inhibition was calculated.

Reagents

- 2.8mM, Deoxyribose.
- 01mM, EDTA.
- 1mM, Hydrogen peroxide.

Nitric oxide radical scavenging activity [5]

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions. The reaction mixture (3ml) containing 2 ml of sodium nitroprusside (10mM), 0.5ml of phosphate buffer saline (1M) were incubated at 25^0C for 150 mins. After incubation, 0.5ml of the reaction mixture containing nitrite was pipetted and mixed with 1 ml of sulphanilic acid reagent (0.33%) and allowed to stand for 5 min for completing diazotization.

Then 1ml of naphthylethylene diamine dihydrochloride (1% NEDA) was added, mixed and allowed to stand for 30 mins. Sodium nitroprusside in aqueous solution at physiological P^H spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions which can be estimated by the use of Griess Illosvery reaction at 540nm.

Reagents

- 10mM, Sodium nitroprusside.
- 1M, Phosphate buffered saline.
- Sulphanilic acid reagent (0.33%).
- Napthylethylene diamine dihydrochloride (1% NEDA).

Total Antioxidant Activity (Phosphomolybdic acid Method)

The antioxidant activity of the sample was evaluated by the transformation of Mo (VI) to Mo (V) to form phosphomolybdenum complex[6]. An aliquot of 0.4 ml of sample solution was combined in a vial with 4 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The vial was capped and incubated in a water bath at 95°C for 90 min. After the samples are cooled to room temperature, the absorbance of the mixture was measured at 695 nm against a blank. The antioxidant activity expressed relative to that of ascorbic acid.

Reagents

- 0.6M Sulfuric acid.
- 28mM Sodium phosphate.
- 4mM Ammonium molybate.

Ferric Reducing Ability Power (FRAP) Assay

A modified method of Benzie and Strain (1996) was adopted for the FRAP assay. The stock solutions included 300mM acetate buffer, pH 3.6, 10mM TPTZ (2, 4, 6-tripyridyl-S-triazine) solution in 40mM HCl and 20mM $FeCl_3.6H_2O$. The fresh working solution was prepared by mixing 25ml acetate buffer, 2.5ml TPTZ and 2.5ml $FeCl_3.6H_2O$. The temperature of the solution raised to 37°C before using. Plant extracts (0.15 ml) were allowed to react with 2.85 ml of FRAP solution for 30 min in the dark condition. Readings of the colored product (Ferrous tripyridyltriazine complex) were taken at 593nm. The standard curve was linear between 200 and 1000 µM $FeSO_4$. Results are expressed in µM (Fe (II) /g dry mass and compared with that of ascorbic acid.

Reagents

- 300mM acetate buffer, pH 3.6.
- 10mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution.
- 40mM HCl.
- 20 mM $FeCl_3.6H_2O$.

Estimation of Total Phenol and Flavonoids Content in Ethanolic Extracts from *Vallaris solanacea*

Total Phenol [7]

2.5ml of various concentration extracts added with 0.5 ml of Folins phenol reagent and 2ml of sodium carbonate (20%). The reaction mixture was kept in boiling water bath for 1 min. the absorbance was measured at 650 nm in a spectrophotometer.

Reagents

- 80% ethanol.
- Sodium carbonates (20%).

Total Flavonoids [8]

0.5 ml of various concentrations of extracts added with 4ml of the vanillin reagent (1% vanillin in 70% conc. H_2SO_4) and kept in a boiling water bath for 15 mins. The absorbance was read at 360 nm. A standard was run by using catechol (110 µg/ml).

Reagents

- 1% Vanillin in 70 % conc. H_2SO_4.

Result and Discussion

The *in-vitro* antioxidant activities of the ethanolic extract of *Vallaris solanacea* studies were determined by following methods namely,

- DPPH assay
- Superoxide anion scavenging activity
- Iron-chelating assay
- Hydroxyl radical scavenging activity
- Nitric Oxide free radical scavenging capacity
- Total antioxidant activity
- FRAP assay

Free radical is a molecule with an unpaired electron and is involved in bacterial and parasitic infections, lung-damage,

inflammation, reperfusion injury, cardiovascular disorders, atherosclerosis, aging and neoplastic diseases [9]. They are also involved in autoimmune disorders like rheumatoid arthritis etc. Antioxidant compounds may function as free radical scavengers, initiator of the complexes of pro-oxidant metals, reducing agents and quenchers of singlet oxygen formation.

Phenolic compounds and flavonoids are major constituents of most of the plants reported to possess antioxidant and free radical scavenging activity. Therefore, the importance of search for natural antioxidants has increased in the recent years so many researchers focused the same.

DPPH Scavenging Activity

DPPH is a stable free radical at room temperature often used to evaluate the antioxidant activity of several natural compounds. The reduction capacity of DPPH radicals is determined by the decrease in its absorbance at 517nm, which is induced by antioxidants. The percentage of DPPH radical scavenging activity of ethanolic extract of *Vallaris solanacea* is presented in Table 1.

The ethanolic extract of *Vallaris solanacea* exhibited a maximum DPPH scavenging activity of 63.88% at 1000 µg/ml whereas for Rutin (standard) was found to be 62.88% at 1000 µg/ml. The IC_{50} of the ethanolic extract of *Vallaris solanacea* and ascorbate were found to be 625µg/ml and 587µg/ml respectively.

Table 1: Effect of ethanolic extract *Vallaris solanacea* on DPPH assay

S. No	Concentration (µg/ml)	% of Activity(±SEM) *	
		Ethanol Extract of *Vallaris solanacea*	Standard (Ascorbate)
1	125	17.67±0.01	20.34±0.30
2	250	34.45±0.04	37.03±0.07
3	500	54.88±0.90	50.33±0.05
4	1000	62.88±0.09	68.33±0.07
		IC_{50}=625 µg/ml	**IC_{50}=587µg/ml**

*All values are expressed as mean ± SEM for three determinations

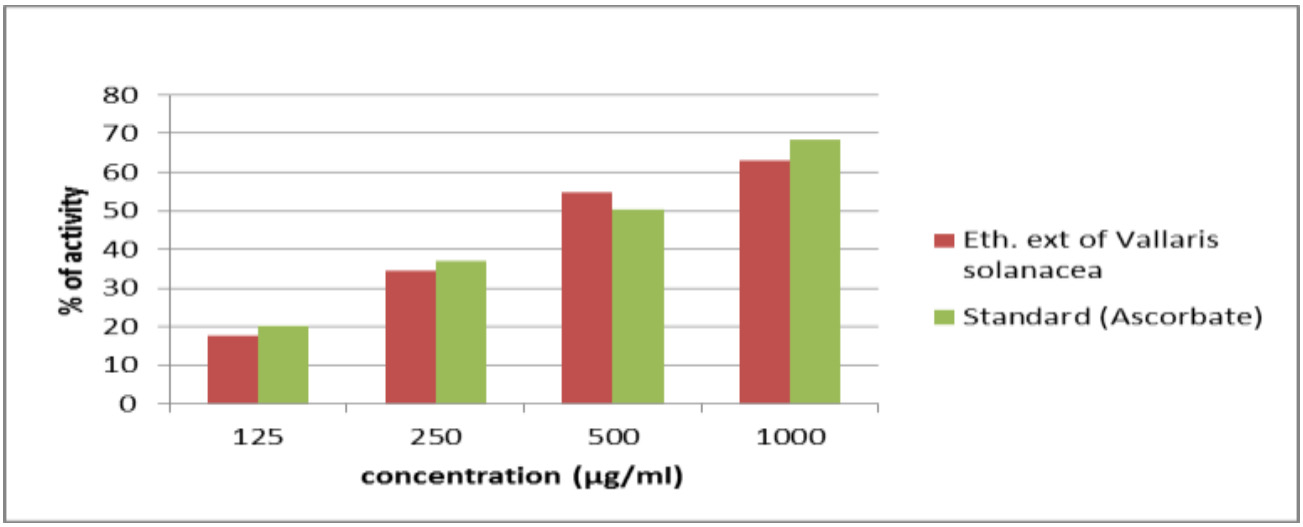

Fig 1: Effect of ethanolic extract of *Vallaris solanacea* on DPPH assay

Superoxide anion scavenging activity

Superoxide is a highly reactive molecule that reacts with various substances produced through metabolic processes. Superoxide dismutase enzymes present in aerobic and anaerobic organisms catalyses the breakdown of superoxide radical [10]. Percentage scavenging of superoxide anion examined at different concentrations of ethanolic extract of *Vallaris solanacea* (125, 250, 500, 1000 µg/ml) is depicted in Table 2.

The percentage scavenging of superoxide radical surged with the enhanced concentration of plant extracts. The maximum scavenging activity of plant extract and Quercetin at 1000 µg/ml was found to be 65.15% and 63.18% respectively.

Superoxide scavenging ability of plant extract might primarily be due to the presence of flavonoids. The IC_{50} value of plant extract and Quercetin was recorded as 459µg/ml and 399µg/ml respectively.

Table 2: Effect of ethanolic extract of *Vallaris solanacea* on Superoxide anion scavenging activity

S. No	Concentration (µg/ml)	% of Activity(±SEM) *	
		Ethanol extract of *Vallaris solanacea*	Standard (Quercetin)
1	125	31.20±0.02	34.44 ± 0.48
2	250	43.63±0.29	47.28 ± 0.49
3	500	61.29±0.70	63.39 ± 0.18
4	1000	65.15±0.05	63.18 ± 0.09
		IC_{50}=459µg/ml	**IC_{50}=399µg/ml**

*All values are expressed as mean ± SEM for three determinations

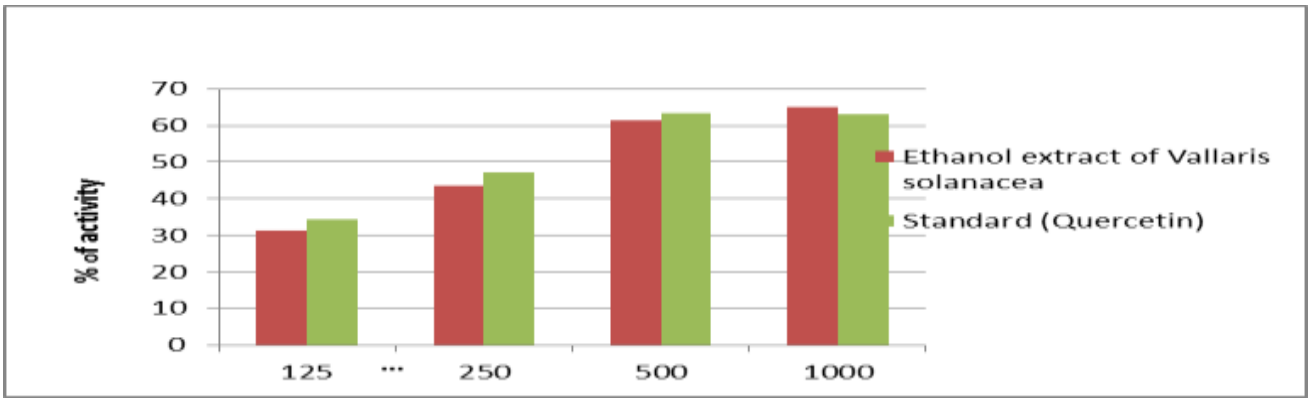

Fig 2: Effect of ethanolic extract of *Vallaris solanacea* on Superoxide anion scavenging activity

Iron chelating activity

Iron is essential for life because it is required for transportation of oxygen, respiration and activity of many enzymes. However, iron is an extremely reactive metal and catalyzes oxidative changes in lipids, proteins and other cellular components [11, 12]. It causes lipid peroxidation through the Fenton and Haber-Weiss reaction [13] and decomposes the lipid hydroxide into peroxyl and alkoxyl radicals that can perpetuate the chain reactions [14]. Iron binding capacity of the ethanolic extract of *Vallaris solanacea* and the metal chelator EDTA at various concentrations (125, 250, 500, 1000 µg/ml) was examined and the values are presented in Table 3. The results of the present study show that the ethanolic extract of *Vallaris solanacea* was found to be most effective and the plant extract possess iron binding capacity which might be due to the presence of polyphenols that averts the cell from free radical damage by reducing of transition metal ions [15, 16]. Various plant extracts were proved to be good chelators [17] and correlation exists between phenols, flavonoids and chelating activity.

Table 3: Effect of ethanolic extract of *Vallaris solanacea* on Iron-chelating method

S. No	Concentration (µg/ml)	% of Activity(±SEM) *	
		Ethanol Extract of *Vallaris solanacea*	Standard (EDTA)
1	125	16.41 ± 0.24	20.02 ±0.07
2	250	37.38 ± 0.24	46.37±0.07
3	500	51.97 ± 0.38	56.89±0.05
4	1000	62.35 ± 0.97	63.38±0.03
		IC_{50}=639µg/ml	**IC_{50}=550µg/ml**

*All values are expressed as mean ± SEM for three determinations

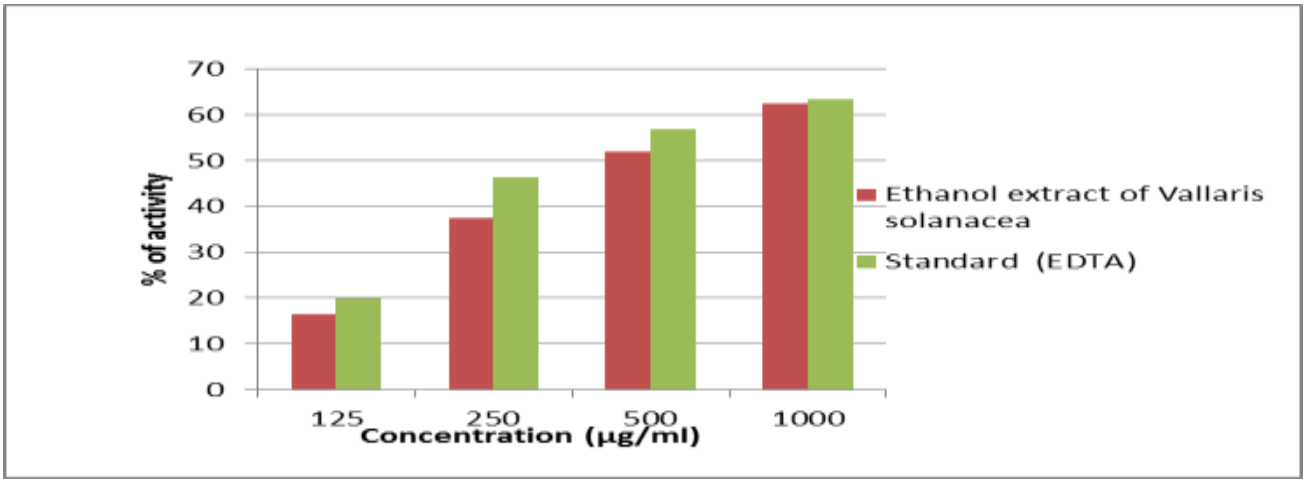

Fig 3: Effect of ethanolic extract of *Vallaris solanacea* on Iron-chelating method

Hydroxyl radical scavenging activity

The highly reactive hydroxyl radicals can cause oxidative damage to DNA, lipids and proteins [18]. The percentage of Hydroxyl radical scavenging activity of ethanolic extract of *Vallaris solanacea* is presented in Table 4 The ethanolic extract of *Vallaris solanacea* was exhibited a maximum Hydroxyl radical scavenging activity of 66.58 % at 500 µg/ml whereas for ascorbate (standard) was found to be 64.54 % at 500 µg/ml. The IC_{50} values of the ethanolic extract of *Vallaris solanacea* and ascorbate were found to be 556µg/ml and 573µg/ml respectively.

Table 4: Effect of ethanolic extract of *Vallaris solanacea*. Hydroxyl radical scavenging activity

S. No	Concentration (µg/ml)	% of Activity(±SEM) *	
		Ethanol extract of *Vallaris solanacea*	Standard (Ascorbate)
1	125	15.01±0.01	24.87 ± 0.08
2	250	34.09±0.02	36.38 ± 0.05
3	500	66.58±0.31	64.54 ± 0.02
4	1000	65.43±0.02	58.63 ± 0.01
		IC_{50}=556 µg/ml	**IC_{50}=573µg/ml**

*All values are expressed as mean ± SEM for three determinations

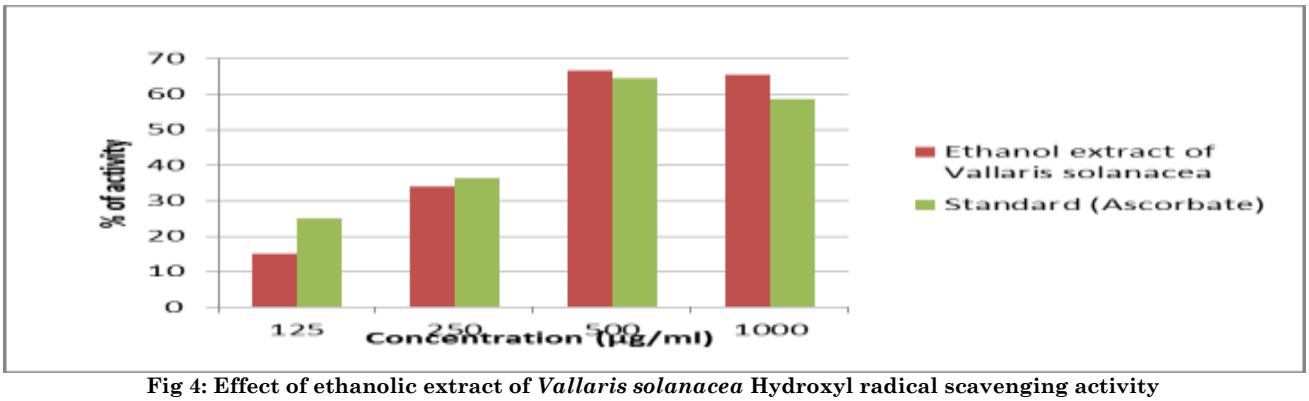

Fig 4: Effect of ethanolic extract of *Vallaris solanacea* Hydroxyl radical scavenging activity

Nitric oxide Scavenging Activity

Nitric oxide is a diffusible free radical which is an important molecule in diverse biological systems [19]. The reduction of nitric oxide radical by the ethanolic extract of *Vallaris solanacea* and ascorbate was noted to be concentration dependent and is illustrated in Table 5. The maximum scavenging activity of ethanolic extract of *Vallaris solanacea* and ascorbate at 1000 µg/ml were found to be 61.49 % and 54.23% respectively. The IC_{50} values of plant extract and ascorbate were recorded as 683µg/ml and 692µg/ml respectively.

Table 5: Effect of ethanolic extract of *Vallaris solanacea*. Nitric oxide radical scavenging activity

S. No	Concentration (µg/ml)	% of Activity(±SEM) *	
		Ethanol extract of *Vallaris solanacea*	Standard (Ascorbate)
1	125	22.24±0.05	26.87 ± 0.08
2	250	27.08±0.01	31.30 ± 0.05
3	500	49.62±0.34	58.64 ± 0.02
4	1000	61.49±0.41	54.23 ± 0.01
		IC_{50}= 683g/ml	**IC_{50}=692µg/ml**

*All values are expressed as mean ± SEM for three determinations

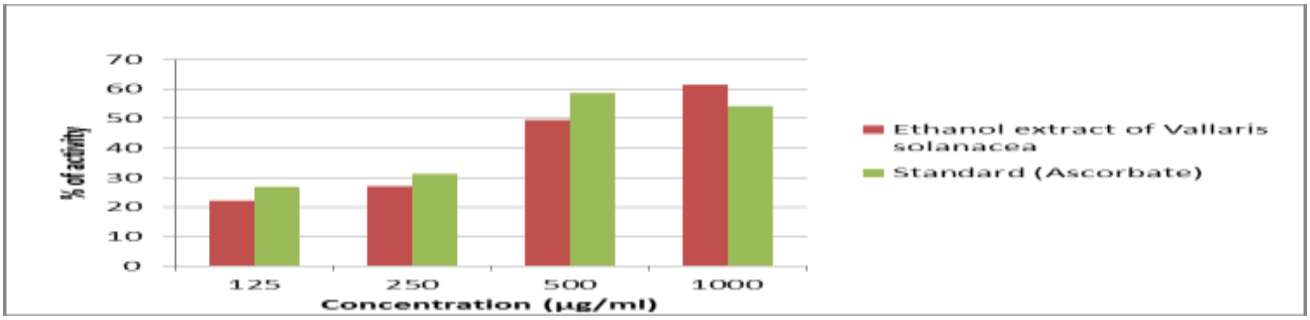

Fig 5: Effect of ethanolic extract of *Vallaris solanacea* Nitric oxide radical scavenging activity

Total Antioxidant Activity (Phosphomolybdic Acid Method)

The percentage of total antioxidant activity of ethanolic extract of *Vallaris solanacea* is presented in Table 6. The ethanolic extract of *Vallaris solanacea* exhibited a maximum total antioxidant activity of 58.16 % at 1000 µg/ml whereas for ascorbate (standard) was found to be 60.64% at 500 µg/ml. The IC_{50} of the ethanolic extract of *Vallaris solanacea* and ascorbate were found to be 638µg/ml and 661µg/ml respectively.

Table 6: Total antioxidant activity of Ethanol extract of *Vallaris solanacea*

S. No	Concentration (µg/ml)	% of activity(±SEM) *	
		Ethanol extract of *Vallaris solanacea*	Standard (Ascorbate)
1	125	21.46±0.05	27.57 ± 0.08
2	250	41.63±0.01	31.30 ± 0.05
3	500	54.36±0.03	60.64 ± 0.02
4	1000	58.16±0.01	55.03 ± 0.01
		IC_{50}=638 µg/ml	**IC_{50}=661µg/ml**

*All values are expressed as mean ± SEM for three determinations

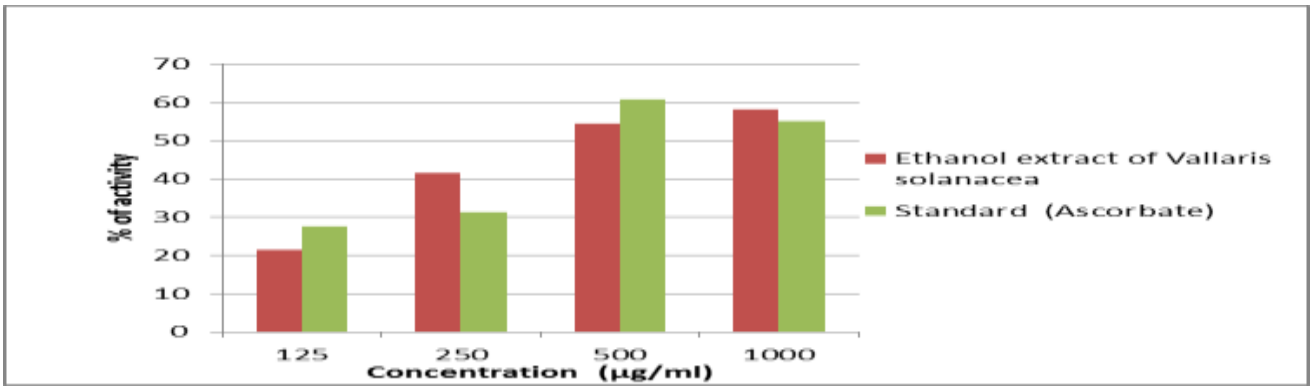

Fig 6: Total antioxidant activity of Ethanol extract of *Vallaris solanacea*

FRAP Assay

The antioxidant potential of *Vallaris solanacea* was ascertained from FRAP assay based on their ability to reduce TPTZ-Fe (III) complex to TPTZ-Fe (II). The reducing ability of the various extract of *Vallaris solanacea* and ascorbate at various concentrations (125, 250, 500, 1000 µg/ml) was examined and the values are presented in Table 7. The maximum reducing ability at 1000µg/ml for ethanolic extract of *Vallaris solanacea* and ascorbate were found to be 65.39% and 61.64 % respectively. The IC_{50} values of ethanolic extract and ascorbate were recorded as 543µg/ml and 575µg/ml respectively.

Table 7: FRAP assay of Ethanol extract of *Vallaris solanacea*

S. No	Concentration (µg/ml)	% of activity (±SEM) *	
		Ethanol extract of *Vallaris solanacea*	Standard (Ascorbate)
1	125	21.56±0.06	26.87 ± 0.08
2	250	44.63±0.01	33.30 ± 0.05
3	500	55.47±0.08	61.64 ± 0.02
4	1000	65.39±0.04	61.23 ± 0.01
		IC_{50}=543 µg/ml	**IC_{50}=575µg/ml**

*All values are expressed as mean ± SEM for three determinations

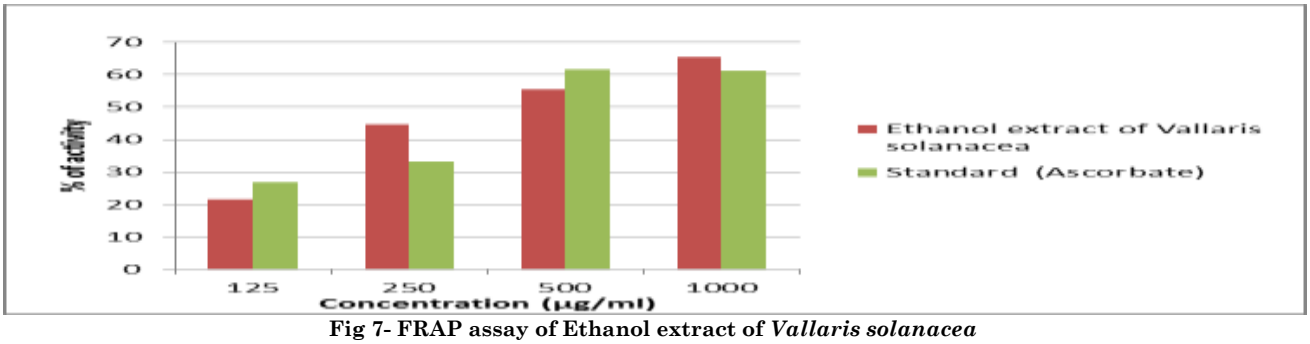

Fig 7- FRAP assay of Ethanol extract of *Vallaris solanacea*

Estimation of total Phenol and flavonoids contents of ethanol extract of *Vallaris solanacea*

Total phenol

Phenolic compounds are known as powerful chain breaking antioxidants. Phenols are very important a plant constituent because of their scavenging ability due to their hydroxyl groups [20].The total amount of phenolic content of ethanolic extract of *Vallaris solanacea* is presented in Table 8 The ethanolic extract of *Vallaris solanacea* was found higher content of phenolic components.

Table 8: the total Phenolic content of ethanolic extract of *Vallaris solanacea*

S. No	Extract	Total phenol content (mg/g) (±SEM) * *oCatechol) (±SEM) *
1	Ethanolic extract of *Vallaris solanacea*	3.980±0.006

*All values are expressed as mean ± SEM for three determinations

Total flavonoids

Flavonoids present in food of plant origin are also potential antioxidants [21, 22]. Most beneficial effects of flavonoids are attributed to their antioxidant and chelating abilities [23]. The total amount of flavonoids content of ethanolic extract of aerial parts of *Vallaris solanacea* is presented in Table 9 the higher content of flavonoids was found in the ethanolic extract of *Vallaris solanacea*.

Table 9: The Total Flavonoids content of ethanolic extract of *Vallaris solanacea*

S. No	Extract	Total flavonoids content (mg/g) (±SEM) *
1.	Ethanolic extract of *Vallaris solanacea*	2.651 ± 0.077

*All values are expressed as mean ± SEM for three determinations

Conclusion

The ethanolic extract of *Vallaris solanacea* leaves was able to reduce the free radical 2-2 diphenyl-1-picryl Hydrazyl (DPPH) to yellow colored diphenyl picryl hydrazine and showed better antioxidant activity with IC50 value of 625µg/ml. Superoxide anion scavenging activity methods shows activity with an IC50 value of 459µg/ml and in hydroxyl radical scavenging activity showed antioxidant activity with IC50 value of 556µg/ml.

Iron chelating activity showed better antioxidant activity with IC50 value of 639µg/ml. Nitric oxide scavenging activity showed Effective antioxidant activity with IC50 value of 683µg/ml. The total antioxidant activity of ethanol extract of *Vallaris solanacea* leaves was determined by phosphomolybdic acid method, it showed antioxidant activity with IC50 value of 638µg/ml. FRAP method radical potential showed effective antioxidant activity with IC50 value of 543µg/ml.

Total phenol content was 3.980±0.006 and respectively the total flavonoids content is 2.651±0.077. The present study has revealed that the ethanol extract of *Vallaris solanacea* leaves contains substantial amount of phenolics and flavonoids thus, can be inferred that these phenolics and flavonoids are responsible for its marked antioxidant activity as assayed through various *in vitro* models used in this study. This is consistent with several reports that have shown close relationship between total phenolic contents, flavonoids and anti-oxidative activity of fruits, plants and vegetables [24-26]. On the basis of the results obtained in the present study it was concluded that the ethanolic leave extracts of this species possess significant antioxidant activity.

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