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Comparative antioxidant studies of *in vivo* leaves and *in vitro* callus of *Crotalaria pallida* Aiton (Fabaceae)

Kiruthiga R, Rakkimuthu R and Aravinthan K M*

Research Department of Biotechnology, Dr. Mahalingam Centre for Research and Development
N. G. M. College, Pollachi, Coimbatore, Tamil Nadu - 642001, India
*Corresponding author email id: k.m.aravinthan@gmail.com

***Crotalaria pallida* Aiton. Fabaceae has been used for the treatment of various diseases. The aim of the present study was to assess the enzymatic and non-enzymatic activities of *Crotalaria pallida* Aiton. The enzymatic antioxidant activity of *in vivo* leaves and *in vitro* callus was compared. The SOD shows high activity of (0.78±0.036) units/g tissue in *in vivo* leaves and Catalase shows high activity of (3.563±0.16) units/g tissue in *in vitro* callus. The non-enzymatic antioxidant properties of *in vivo* leaves and *in vitro* callus were evaluated using multiple assays. DPPH, Hydroxyl scavenging activity and Superoxide ion radical scavenging activity of *in vivo* leaves and *in vitro* callus were exhibited the IC_{50} values of 25±0.16 and 23.57±0.07, 90.18±3.57 and 77.41±2.5, 72.58±1.55 and 42.96±0.29 respectively. The total antioxidant activity showed increase with increase in concentration of both *in vivo* leaves and *in vitro* callus. The obtained results indicate that *in vivo* leaves and *in vitro* callus of *Crotalaria pallida* Aiton posses potent enzymatic activity and is a good source of non enzymatic antioxidants.**

Key words: *Crotalaria pallida*, enzymatic measurement, antioxidant potential, radical quenching, DPPH assay.

INTRODUCTION

Plants, our gift of nature which is the sources of bioactive constituents have been used traditionally to cure various ailments in Ayurvedha, Unani and Siddha. During last few years, synthetic drugs occupy the position for curing various diseases. Due to their side effects, scientists are now focusing to explore the potentiality of traditional medicines. In healthy individuals, the production of free radicals is balanced by the antioxidative defence system (Halliwell, 1997).

Crotalaria is one of the largest genera in tropical Africa. The genus includes 690 species that are mainly situated in Africa and Madagascar (Roux *et al.*, 2009). Species have also been found in India, United States of America (USA) and China. *Crotalaria pallida* Aiton. belongs to the family fabaceae. This is an erect shrub, annual or short-lived perennial herb of 1.5 m or more tall. The plant is grown as a ground cover and a green manure crop, especially in the inter-rows of rubber trees and coconut palms. Flowers are eaten as a vegetable in Cambodia, where the seeds are roasted and grounded for use as a sort of coffee beverage. The roots are sometimes

chewed with betel nuts in Vietnam. In traditional medicine, the plant is used to treat urinary problems and fever, a poultice of the roots is applied to swelling of joints and an extract of the leaves is taken to expel intestinal worms (Chong and Corlett, 2009).

Due to prooxidative enzyme systems, lipid oxidation, irradiation, inflammation, smoking, air pollutants and glycoxidation, reactive oxygen species or free radicals (superoxide, hydroxyl, hydrogen peroxide) are highly produced and exceeds the normal enzymatic (superoxide dismutase, catalase, glutathione peroxidase) and non-enzymatic antioxidants (ascorbic acid, α- tocopherol, glutathione, carotenoids and flavonoids) in the body. Hyper physiological burden of free radicals causes the imbalance between free radicals and antioxidants and results in the oxidation of biomolecules (protein, amino acids, lipids and DNA) (i.e., oxidative stress) and lead to cell injury and death (Halliwell, 1997).

This have been implicated in a number of degenerative diseases like atherosclerosis, diabetes mellitus, ischemia/reperfusion (I/R) injury, Alzheimer's disease, inflammatory diseases, carcinogenesis, neurodegenerative diseases, hypertension, ocular diseases, pulmonary diseases and haematological diseases (Maxwell, 1995). Hence, there is a need to supply exogenous supply of antioxidants, where synthetic antioxidants like butylated hydroxylanisole (BHA), butylated hydroxytoluene (BHT) and tertbutylated hydroquinone (TBHQ) were reported to be carcinogenic (Botterweck *et al*., 2000).

MATERIALS AND METHODS

Plant material

The leaves of *C. pallida* Aiton were procured from natural vicinity of Pollachi town, Coimbatore city, India during the month of April 2013. The plant was identified and authenticated (410) by Botanical Survey of India, TNAU campus, Coimbatore, Tamilnadu, India, as *Crotalaria pallida* Aiton.

Sample extraction for enzymatic assay

One gram of each fresh leaf tissue (*in vivo)* and callus (*in vitro)* were weighed and ground in a chilled mortar and pestle with buffer solution pH-7.0 for superoxide dismutase, catalase, guiacol peroxidase activity and buffer solution pH- 6.5 for ascorbic acid oxidase activity respectively. The extract was centrifuged at 4˚C for 10 min at 10,000 rpm. The supernatant obtained was used for the determination of enzymatic assays.

Chemicals

Hydrogen peroxide (H_2O_2), Guaiacol, Ascorbic acid, Butylated hydroxytoluene(BHT), 2,2'- diphenyl – 1- picryl-hydrazyl (DPPH), Ascorbic acid, Sulphuric acid, Sodium phosphate, Ammonium molybdate, Ethylene diamine tetra acetic acid(EDTA), Trichloroacetic acid (TCA), DMSO solution, Riboflavin, Methionine, Hydroxylamine, Nitroblue tetrasolium chloride(NBT), Ferrous ammonium sulphate were obtained from Hi media. All chemicals used were of analytical grade.

Enzymatic antioxidants

Antioxidant activity of some of the enzymes such as superoxide dismutase, catalase, peroxidase and ascorbic acid oxidase were studied in the leaf extract and callus.

Estimation of total protein

The total protein was estimated by the method of Lowry *et al.,* (1951). To different concentrations of standards, 0.01 ml of leaves extract (*in vivo*) and callus extract (*in vitro*) were added separately and the volume was made upto 1 ml with distilled water. To all the tubes 5ml of alkaline copper sulphate reagent was added and left at room temperature for 10 min. Then 0.5 ml Folin ciocalteau reagent was added and incubated at room temperature for 20 min. The colour developed was read at 660 nm and the protein concentration was expressed as mg per gm extract.

Assay of Superoxide dismutase

Assay of superoxide dismutase was carried according to the method of Beauchamp and Fedovich (1976). To 0.5 ml of leaves and callus extract, 1ml of 125mM sodium carbonate, 0.4ml of 25µM NBT and 0.2ml of 0.1mM EDTA were added separately. The reaction was initiated by adding 0.4ml of 1mM hydroxylamine hydrochloride and the absorbance was read at 560nm using spectrophotometer at 5min intervals. Units of SOD were expressed as amount of enzyme required for inhibiting the reduction of NBT by 50%. The specific activity was expressed in terms of units per mg of protein.

Assay of Catalase

The method of Luck (1963), as mentioned in sadasivam and manikam (1991) was adopted to measure the activity of catalase. The leaves and callus extract (0.1ml) was added to the reaction mixture containing 3ml of H_2O_2 and 0.01M phosphate buffer (pH 7.0) separately and the OD change was measured at 240nm, the time taken for decrease in the absorbance from 0.45 to 0.4 is noted as GT. The activity of the enzyme is expressed in the terms of F mole of H_2O_2 consumed/ min/ mg protein. The activity of catalase was calculated by the following formula,

$$\text{Units in the assay mixture} = 17/\Delta T$$

ΔT is the time in seconds.

Assay of Guaicol peroxidase

The assay was carried out by the method of Putter (1974). The reaction mixture consisted of 3ml of assay buffer (0.1M phosphate buffer, (pH 7.0), 20mM guaiacol, and 0.03 ml of 30% H_2O_2). To this 0.1ml of leaves extract (*in vivo*) and callus (*in vitro*) extract was added separately and OD change was measured at 436 nm. The peroxidase activity was calculated using an extinction coefficient of guaiacol dehydrogenase (liters /mol). The activity of guaicol peroxidase was calculated by the formula,

$$\text{Guaicol peroxidase activity units/litre} = \frac{3.18\times0.1\times1000}{6.39\times1\times\Delta T\times0.1}$$

Where 0.1=volume of sample; 6.39=extinction coefficient; Δt=time in minutes.

Assay of Ascorbic acid oxidase

Assay of ascorbic acid oxidase activity was carried out according to the procedure of oberbacher and vines (1963). The 3.0ml of the substrate solution (8.8mg of ascorbic acid in 300ml phosphate buffer, pH(5.6). 0.1ml of the leaves extract (*in vivo*) and callus extract (*in vitro*) was

added separately and the absorbance change was measured at 265nm for every 30second for a period of 5minutes. One enzyme unit is equivalent to 0.01OD change per minutes.

Non enzymatic assays
Antioxidant ability analyses
Sample extraction for non-enzymatic assay

About 30g of powdered leaves (*in vivo*) and callus (*in vitro*) sample of *C. pallida* Aiton was dissolved in 300ml of methanol and kept in an orbital shaker for overnight. The extract was filtered using Whatman filter No.1 to obtain a particle free extract. The solvent extract was evaporated to dryness after extraction under reduced pressure using rotary evaporator. The obtained methanol extract was stored at 4ºC for further analysis.

Sample preparation

Evaporated *C.pallida* leaves (*in vivo*) and callus (*in vitro*) sample 50mg was dissolved in 1 ml of methanol. From the above stock solution 25, 12.5, 6.25 and 3.125 mg/ml concentration of sample was used for different assays. A standard (Ascorbic acid (or) BHT) was used for the positive control.

Total antioxidant activity

The assay was based on the reduction of Mo(VI)-Mo(V) by the extracts and subsequent formation of a green phosphate/Mo(V) complex at acidic pH (Prieto *et al.*,1999). Different concentrations of methanolic leaves and callus extract of *Crotalaria pallida* (3.125-50 mg/ml) were mixed with 3 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) separately. The tubes were incubated at 95ºC for 90 min. The mixture was cooled to room temperature, and then the absorbance of the solution was measured at 695 nm against blank. The total antioxidant activity was expressed as ascorbic acid equivalent (AAE) in milligrams of ascorbic acid per gm of extract.

DPPH radical scavenging activity

The free radical scavenging activity evaporated leaves and callus samples of *Crotalaria pallida* Aiton. was measured using DPPH as a free radical (Mensor *et al*, 2001). The reaction mixture contained 0.1ml of different concentration of methanolic leaves and callus extract (3.125-50 mg/ml) with 1.9ml of 0.1mM DPPH in methanol separately. The control was devoid of the sample. BHT was used as a positive control. The reaction mixture was shaken well and incubated at 25ºC for 30 min. After incubation, the absorbance was read at 517nm (Genesis-5 UV-Visible spectrophotometer). The percentage of DPPH radical scavenging activity was calculated from the following equation.

DPPH Scavenging activity (%) = $1 - \frac{As}{Ac} \times 100$

Where Ac was the absorbance of control reaction and As the absorbance in the presence of *C. pallida* Aiton leaves and callus extracts.

Hydroxyl radical scavenging activity

The reaction mixture contained 0.1ml of different concentration of methanolic leaves and callus extract (3.125-50 mg/ml) of *C. pallida* Aiton was mixed with 1 mL of iron – EDTA solution (0.13 % ferrous ammonium sulphate in 0.26 % EDTA), 0.5 mL of 0.018 % EDTA and 1 mL of

DMSO solution (0.85 % in 0.1 M phosphate buffered saline, pH=7.4) separately. The reaction was initiated by the addition of 0.5 mL of 0.22 % ascorbic acid and incubated at 80–90^{o} C in water bath for 15 min. The reaction was terminated by the addition of 1 mL of ice cold TCA (17.5 %). Nash reagent (3 mL) was added to the above mixture and allowed to stand at room temperature for 15 min and the absorbance values were recorded at 412 nm (Klein *et al*, 1981) using spectrophotometer. Sample control was also run with the substitution of phosphate buffer instead of ascorbic acid. Reaction mixture without samples was used as control. The percent of hydroxyl radical scavenging activity (HRSA) was calculated using the following formula,

$$\text{HRSA \%} = \frac{[(A \text{ of sample} - A \text{ of Control})}{A \text{ of control}} \times 100$$

Superoxide anion radical scavenging activity

The assay depends on the capacity of different concentration of *C.pallida* Aiton methanolic leaves and callus extract to inhibit formazan formation by scavenging the superoxide radicals generated in riboflavin-light- NBT system (Beauchamp and Fridovich, 1971). The reaction mixture contained 50mM phosphate buffer, pH-7.6, 20µg riboflavin, 12mM EDTA, NBT 0.1mg/3ml, added in sequence. Different concentrations of *C. pallida* Aiton methanolic leaves and callus extract (3.125-50 mg/ml) were added to the reaction mixture separately and the reaction was initiated by illuminating the tubes under fluroscent lamp (20w) for 15 minutes. Immediately after illumination, the absorbance was measured at 560nm using spectrophotometer. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. Identical tubes, with reaction mixture, were kept in the dark and served as blanks. The percentage inhibition of superoxide anion generation was calculated using the following equation.

$$\text{Super oxide radical scavenging capacity (\%)} = 1 - \frac{As}{Ac} \times 100$$

Where Ac was the absorbance of control reaction and As the absorbance in the presence of *C. pallida* Aiton leaves and callus extracts.

Statistical analysis

For all the experiments, the samples were analyzed and all the assays were carried out in triplicate. The results were expressed as mean±standard deviation. The IC_{50} value was calculated by using MS-EXCEL software and standard deviation (SD) was calculated by Regression Co-efficient.

RESULTS

Antioxidant enzyme measurements

Proteins are Macromolecules that act as alternate energy source when other energy sources are in short supply. They are the building block of any organism. The fresh leaves *(in vivo)* and callus *(in vitro)* of *Crotalaria pallida* were analyzed for its protein content and the results obtained are represented in **Table 1**.

Assay of Superoxide dismutase

SOD is the most indispensable enzyme for protecting the cells from the toxicity of the reactive oxygen species that are generated during aerobic respiration for energy production; it converts more toxic superoxide anion radicals to less toxic hydrogen peroxide (Vangronsveld

and Clijsters, 1994). The SOD activity was determined at 560nm. **Table 2** shows the SOD activity of leaves *(in vivo)* and callus *(in vitro)* of *C. pallida*. The activity observed for callus *(in vitro)* 0.826±0.025 Units/g tissue is significant than activity observed in leaves *(in vivo)* 0.78±0.036 Units/g tissue **(Figure 1)**.

Table 1: Protein content of *Crotalaria pallida* Aiton.

Sample	Concentration µg/g of tissue
Leaves (*in vivo*)	39
Callus (*in vitro*)	70

Table 2: Antioxidant enzyme measurements

Activity	Leaves (units/g tissue)	Callus (units/g tissue)
Superoxide dismutase activity	0.78±0.036	0.826±0.025
Catalase activity	0.434±0.021	3.563±0.16
Ascorbic acid oxidase activity	0.0017±0.0002	0.0054±0.00025
Peroxidase activity	0.229±0.02	0.453±0.04

Mean ± S.D, n=3.

Assay of Catalase

Catalases bring about the decomposition of H_2O_2 produced from the photorespiration and ß-oxidation of fatty acids (Beevers, 1979; Huang, 1983). The catalase activity was determined at 240nm. The Catalase activity of leaves *(in vivo)* and callus *(in vitro)* of *C. pallida* obtained is tabulated in **Table 2**. The activity observed for callus *(in vitro)* 3.563±0.16 Units/g tissue is significant than activity observed in leaves *(in vivo)* 0.434±0.021 Units/g tissue **(Figure 1)**.

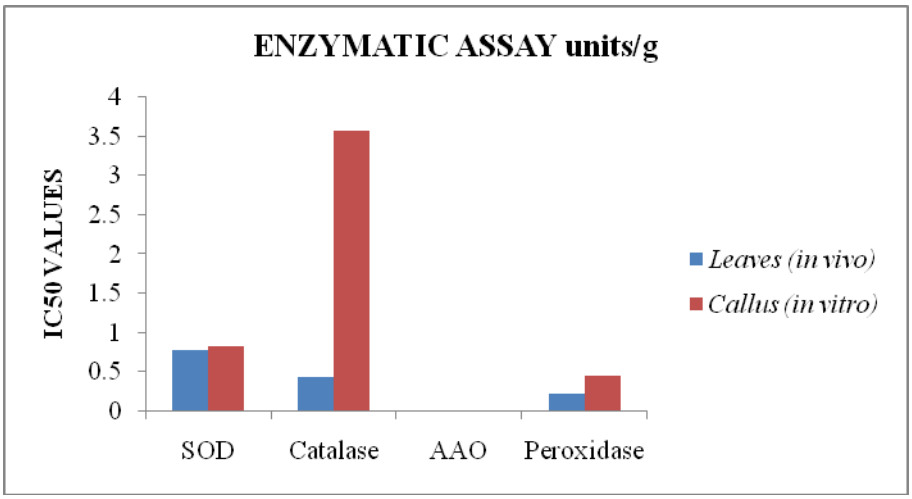

Figure 1: Average values of enzymatic assays of methanolic leaves and callus extracts of *Crotalaria pallida* Aiton (Fabaceae).

Assay of Guaicol peroxidase

The guaicol peroxidase activity was determined at 436nm. The guaicol peroxidase activity of leaves (*in vivo*) and callus (*in vitro*) of *C. pallida* was observed **(Table 2)**. The activity observed for callus *(in vitro)* 0.453±0.04 Units/g tissue is significant than activity observed in leaves *(in vivo)* 0.229±0.02 Units/g tissue **(Figure 1)**.

Assay of Ascorbic acid oxidase

Ascorbic acid oxidase participates in redox reactions involving ascorbic acid (Weis, 1975). The Ascorbic acid oxidase activity of leaves *(in vivo)* and callus *(in vitro)* of *C. pallida* is presented in **Table 2**. The activity observed for callus *(in vitro)* 0.047±0.003 Units/g tissue is significant than activity observed in leaves *(in vivo)* 0.016±0.002 Units/g tissue **(Figure 1)**.

Determination of non-enzymatic antioxidants

Total antioxidant capacity

The total antioxidant capacity of the plant extracts was determined by the phosphomolybdenum method. This method is based on the reduction of molybdenum Mo (VI) to Mo (V) by the antioxidant compounds and the formation of a green Mo (V)-antioxidant complex with maxi-mum absorption at 695 nm. The methanolic leaf and callus extracts of *C. pallida* Aiton. were compared with ascorbic acid. **Figure 2** shows a linear increase in absorbance with increase in concentration of both leaves and callus.

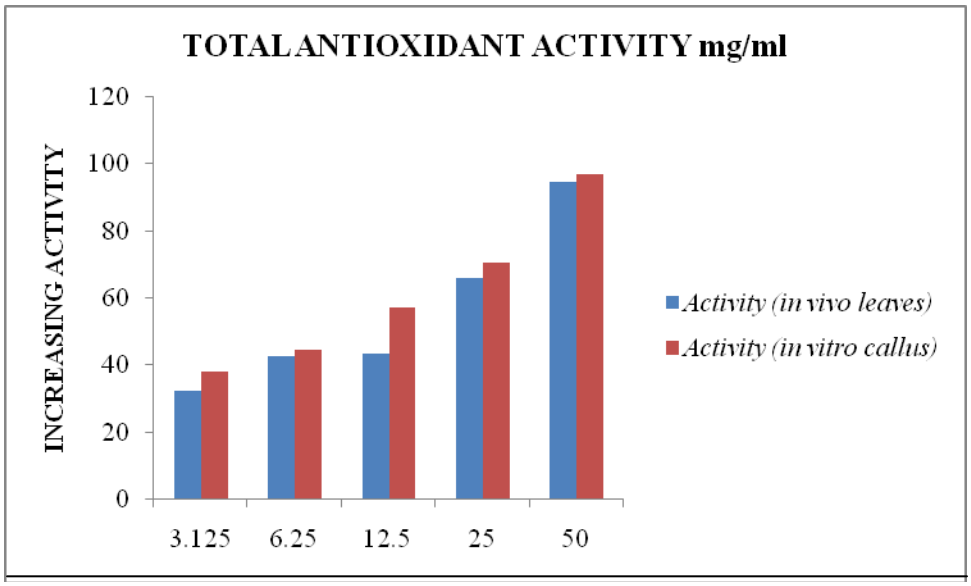

Figure 2: Increasing antioxidant activity of methanolic leaves and callus extracts of *Crotalaria pallida* Aiton (Fabaceae).

DPPH Free Radical Scavenging Assay

The DPPH radical scavenging assay is a sensitive method for the antioxidant screening of Plant extract (Hemaltha *et al*, 2010). The reduction capability of DPPH radicals was determined by decrease in its absorbance at 517nm, which is induced by antioxidants. The percentage of DPPH radical scavenged by BHT, methanolic leaves and callus extracts of *C.*

pallida Aiton. at different concentrations (3.125-25 mg/ml) were presented in **Table 3**. The IC_{50} value was found to be 25±0.16, 23.57±0.07 and 21.55±0.17 for methanolic leaf and callus extracts of *C. pallida* Aiton. and BHT respectively **(Figure 3)**.

Table 3: DPPH radical scavenging activities of methanolic leaves and callus extracts of *C. pallida* Aiton

Concentration	3.125mg/ml	6.25mg/ml	12.5mg/ml	25mg/ml	50mg/ml	IC_{50} Value
Percentage of inhibition (*in vivo* leaves)	16.41±0.33	35.03±0.40	62.46±0.51	72.91±0.77	75.68±0.63	25±0.16
Percentage of inhibition(*in vitro* callus)	19.17±0.32	40.76±0.49	69.06±0.14	73.51±0.42	80.95±0.39	23.57±0.07
Standard (BHT)	45.63±0.44	63.16±0.56	80.67±0.97	81.88±0.92	82.62±0.60	21.55±0.17

Mean ± S.D; n=3

Hydroxyl radical scavenging activity

Hydroxyl radicals are the major active oxygen species formed from the reaction of various hydroperoxides with transition metal ions and attack cell constituents including lipids, nucleic acid and proteins giving rise to many diseases including arthritis, atherosclerosis, cirrhosis, diabetes, cancer, Alzheimer's disease, emphysema and ageing (Welch *et al*, 2002). The scavenging activity of hydroxyl radicals was determined at 412nm. **Table 4** Shows the percentage of hydroxyl radical scavenged by BHT, methanolic leaf and callus extracts of *C. pallida* Aiton. at different concentrations (3.125-25 mg/ml). The IC_{50} value was found to be 90.18±3.57, 77.41±2.57 and 31.71±0.29 for methanolic leaf and callus extracts of *C. pallida* Aiton and BHT respectively.

Table 4: Hydroxyl radical scavenging activity of methanolic leaves and callus extracts of *C. pallida* Aiton.

Concentration	3.125mg/ml	6.25mg/ml	12.5mg/ml	25mg/ml	50mg/ml	IC_{50} Value
Percentage of inhibition(*in vivo* leaves)	5.51±0.33	12.5±0.55	15.83±0.80	18.81±0.89	21.72±0.75	90.18±3.57
Percentage of inhibition(*in vitro* callus)	9.95±1.08	15.12±0.54	18.17±1.04	21.62±0.46	25.21±0.85	77.41±2.5
Standard (BHT)	42.54±0.41	46.7±0.72	50.98±0.83	53.25±0.57	57.15±0.57	31.71±0.29

Mean ± S.D; n=3.

Superoxide anion radical scavenging assay

Superoxide anion radical ($O2^{\cdot}$), a highly toxic radical generated first after oxygen is taken into living cells by several enzymatic and nonenzymatic pathways, plays important role in the formation of other deleterious reactive oxygen species (hydrogen peroxide and hydroxyl) attacks a number of biological molecules and leads to unfavourable alterations of biomolecules including DNA (Waris and Alam, 2004). The capacity of superoxide scavenging activity was determined at 560nm. The percentage of hydroxyl radical scavenged by BHT, methanolic leaf and callus extracts of *C. pallida* Aiton at different concentrations (3.125-25 mg/ml) were

calculated **(Table 5)**. The IC_{50} value was found to be 72.58±1.55, 42.96±0.29 and 37.23±0.39 for methanolic leaf and callus extracts of *C. pallida* Aiton and BHT respectively **(Figure 3)**.

Table 5: Superoxide anion radical scavenging activity of methanolic leaves and callus extracts of *C. pallida* Aiton.

Concentration	3.125mg/ml	6.25mg/ml	12.5mg/ml	25mg/ml	50mg/ml	IC_{50} Value
Percentage of inhibition(*in vivo* leaves)	9.69±0.55	11.40±0.16	20.92±0.06	23.72±0.20	26.8±0.94	72.58±1.55
Percentage of inhibition(*in vitro* callus)	9.02±0.95	29.06±0.29	33.82±0.39	41.98±0.54	43.89±0.09	42.96±0.29
Standard (BHT)	34.92±0.25	37.9±0.36	41.47±0.36	44.83±0.92	49.73±0.47	37.23±0.39

Mean ± S.D; n=3.

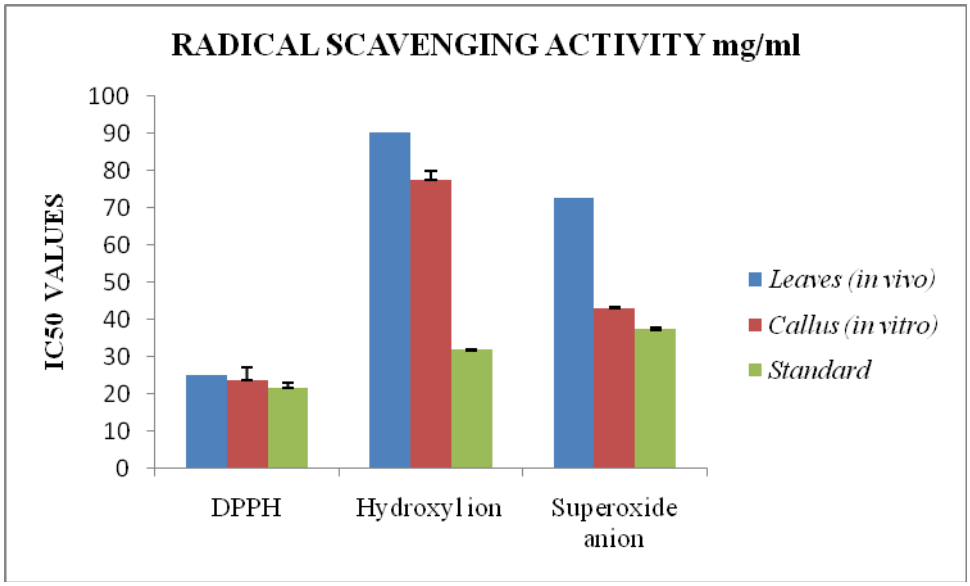

Figure 3: IC_{50} value of Radical scavenging assays of methanolic leaves and callus extracts of *Crotalaria pallida* Aiton (Fabaceae).

DISCUSSION

Nowadays, research is going in around the world to exploit the traditional medicinal plants for therapeutic value scientifically. The qualities like low toxicity, inexpensive and potent pharmacological activities made medicinal plants very useful to mankind. Most of the natural products contain abundant and potent antioxidants. They exert their beneficial effects through the additive action of several chemical compounds acting at single or multiple target sites associated with the physiological process in contrast to synthetic antioxidants based upon a single chemical. The antioxidant activity of the antioxidants have been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging (Oktay *et al*, 2003).

This study is approached with multiple assays to evaluate enzymatic and non-enzymatic capacity of different concentration of *Crotalaria pallida* Aiton. leaves *(in vivo)* and callus *(in vitro)* adapting four enzymatic methods such as superoxide dismutase, catalase, peroxidase, ascorbate oxidase and four non- enzymatic methods such as DPPH, total antioxidant activity, Superoxide ion radical scavenging activity and Hydroxyl ion radical scavenging activity.

Super oxide radicals are inactivated by the enzyme superoxide dismutase (SOD), the only enzyme known to use a free radical as a substrate. The radical scavenging activity of SOD is effective only when it is followed by increase in activity of catalase and other peroxidases (Ramasarma, 1990). SOD is the most indispensable enzyme for protecting the cells from the toxicity of the reactive oxygen species that are generated during aerobic respiration for energy production; it converts more toxic superoxide anion radicals to less toxic hydrogen peroxide (Vangronsveld and Clijsters, 1994). Superoxide dismutase (SOD) activity was found to be significantly higher in *Cassia fistula* (2.29 ± 0.325 units/g dry tissues) (Umesh Kumar *et al*., 2012). *Cassia fistula* [methanol] fraction has exhibited its highest scavenging activity of about 78.2±0.23% in 2 mg/mL. The very next significant activity was presented by *Cassia auriculata* [methanol] and *Cassia fistula* [ethyl acetate] crude of about 73±0.09% and 71±0.16% respectively (Deepika and Sujatha, 2013). The highest SOD activity was found in the *Cucumis melon* varieties such as Galia C8, Midyat and Semame varieties (377.67, 357.00 and 350.00 µMol min-1 mg-1 FW, respectively) on the 8th day callus culture (Sebnem Kusvuran *et al*., 2012). Superoxide dismutase activity of *C. pallida* fresh leaves *(in vivo)* and callus *(in vitro)* exhibited 0.78±0.036 and 0.826±0.025 units/g tissue respectively. Therefore fresh leaves of C. pallida *(in vivo)* and callus *(in vitro)* has less Superoxide dismutase activity than the dry tissue of *Cassia fistula* plant and Cassia fistula [methanol] fraction, Cassia auriculata [methanol] and Cassia fistula [ethyl acetate] crude and *Cucumis melon* varieties. Callus *(in vitro)* of *C. pallida* shows more Superoxide dismutase activity than *C. pallida* fresh leaves *(in vivo)*.

Catalase is a tetrahedrical protein, constituted by four heme groups which catalyze the dismutation of hydrogen peroxide in water and oxygen (Scandalios, 1987). High catalase activity was observed in F1, F2 and F9 leaf extracts of *Ficus deltoidea* accessions with 0.230, 0.203 and 0.064 nmol/min/mg/g respectively (Mansor Hakiman and Mahmood Maziah, 2009). Catalase activity was observed to be significantly higher in *Cassia fistula* (1.196 ± 0.065 units/g dry tissue) (Umesh Kumar *et al*., 2012). The highest catalase activity occurred in *Cucumis melon* callus varieties - Midyat with a value of 226.02 µMol/min-1 mg-1 FW (Sebnem Kusvuran *et al*., 2012). Catalase activity of *C. pallida* fresh leaves (in vivo) and callus (in vitro) exhibited 0.434±0.021 and 3.563±0.16 units/g tissue respectively. Therefore *C. pallida* fresh leaves *(in vivo)* and callus *(in vitro)* has more catalase activity than *Ficus deltoidea* accessions (female leaves) and *C. pallida* fresh leaves *(in vivo)* has less catalase activity than dry tissue of *Cassia fistula* plant and *C. pallida* callus *(in vitro)* has more catalase activity than dry tissue of *Cassia fistula* plant and less activity than callus culture of Midyat variety of *Cucumis melon*. *C. pallida* callus *(in vitro)* shows more catalase activity than *C. pallida* fresh leaves *(in vivo)*.

Peroxidases are referring to heme containing enzymes which are able to oxidise organic and inorganic compounds using hydrogen peroxide as co-substrate. The non-specificity of peroxidase makes the enzyme suitable to a broad range of electron donor substrates (Halliwell, 2006). The highest peroxidase activity showed in F13 leaf extract of *Ficus deltoidea* accessions (female leaves) with 0.94 nmol / min / mg/g (Mansor Hakiman and Mahmood Maziah, 2009).

Crude potassium phosphate buffer extracts from *Pothomorphe umbellate* callus (30 days old) showing peroxidase activity have small values of k_M, indicating very good specificity of the enzyme for guaiacol substrate, showed by the values of k_M and v_{max}, 3.3 mmol.L-1 and 2.8 sec-1, respectively (Khalil *et al*., 2006). Crude extract of root-derived callus and shoot-derived callus of *Moringa oleifera* possessed specific activity of 167.25 ± 16.12 and 103.99 ± 10.64 unit/mg proteins (Lalida *et al*., 2013). The guaicol peroxidase activity of *C. pallida* fresh leaves *(in vivo)* and callus *(in vitro)* exhibited 0.229±0.02 and 0.453±0.04 units/g tissue respectively. Therefore *C. pallida* fresh leaves *(in vivo)* and callus *(in vitro)* has less guaicol peroxidase activity than F13 leaf extract of *Ficus deltoidea* accessions (female leaves). *C. pallida* callus *(in vitro)* shows more guaicol peroxidase activity than *C. pallida* fresh leaves *(in vivo)*.

According to Murata *et al*. (2008), ascorbate oxidase is a member of the multicopper oxidase family and can be obtained from higher plants such as green zucchini squash and cucumber and fungal species. Ascorbate oxidase catalyzes the one-electron oxidation of ascorbate with the concomitant four-electron reduction of dioxygen to water (Solomon *et al*., 1996). F1 leaf extract of *Ficus deltoidea* accessions (female leaves) showed the highest ascorbate oxidase activity with 24.64 nmol/min/mg/g (Mansor and Mahmood, 2009). Jasmonic acid in *Cucumis melon* cell suspension culture at 10 µMol concentrations induced highly significant ascorbic acid oxidase activity of average 207.9, 592.3 and 79.2% increase respective to interval harvesting time. In contrast JA at 0.5 and 5 µMol concentrations induced significant average 50, 7.3% lower than the control respectively at 72 h harvesting time (Eetezaz Nafie *et al*., 2011). The Ascorbic acid oxidase activity of *C. pallida* fresh leaves (*in vivo*) and callus (*in vitro*) exhibited 0.0017±0.0002 and 0.0054±0.00025 respectively. Therefore *C. pallida* fresh leaves *(in vivo)* and callus *(in vitro)* has less Ascorbic acid oxidase activity than F1 leaf extract of *Ficus deltoidea* accessions (female leaves) and Cucumis melon cell suspension culture. *C. pallida* callus (in vitro) shows more Ascorbic acid oxidase activity than *C. pallida* fresh leaves *(in vivo)*.

The DPPH radical has been used widely to test the potential of the compounds as free radical scavengers of hydrogen donors and to investigate the antioxidant activity of plant extracts. The DPPH free radical scavenging activity is due to the neutralization of DPPH free radical by extract either by transfer of hydrogen or of an electron (Shimada *et al*, 1992). Free radical-scavenging is one of the known mechanisms by which antioxidants inhibit the lipid oxidation (Amarowicz *et al*, 2004).The concentraction of the sample of the leaves extract to decrease initial concentraction of DPPH by 50% (IC_{50}) was determined. The lower value of IC_{50} indicates a higher antioxidant activity. DPPH scavenging activity of methanolic leaves and callus extracts of *C. pallida* exhibited 75.68% and 80.95% at 50 mg/ml concentration with IC_{50} value of 25±0.16 mg/ml, 23.57±0.07 mg/ml respectively and IC_{50} value of 21.55±0.17 mg/ml for BHT (STD). DPPH scavenging activity of ethanol extract of *Crotalaria pallida* Aiton exhibited above 80% scavenging activity at 0.1mg/ml concentration while petroleum ether, ethyl acetate, chloroform and water extracts reached 60% at same concentration (Govindappa *et al*, 2011). The IC_{50} value of DPPH scavenging activity was shown by ethyl acetate leaf extract of *Indigofera tinctoria* L. is 87.77±4.9µg/mL followed by methanolic leaf extract 99.53±0.9µg/mL (Nagarajan anusya and Sellamuthu manian, 2013). Hyper antioxidant activity was observed in one month old callus of *Ipomea aquatica* produced by NAA in combination with kinetin. The EC_{50} value of callus extract was 38±3.05µg/mL for DPPH activity (Nagendra Prasad *et al*., 2005). Ethyl acetate fraction of *Vigna unguiculata* callus (methanol extract) possessed highest DPPH radical scavenging activity as it had minimum IC_{50} value of 80 ±1.2 µg/ml (Sharad Vats, 2012).So methanolic leaves extract *(in vivo)* and methanolic callus extract *(in vitro)* of *C. pallida* has more

significant antioxidant activity than petroleum ether, ethyl acetate, chloroform and water extracts of *C. pallida*. IC_{50} value shows that *C. pallida* has more significant antioxidant activity than ethyl acetate leaf extract of *Indigofera tinctoria* followed by methanolic leaf extract and IC_{50} value of callus of *C. pallida* shows significant activity than callus of *Ipomea aquatic* and *Vigna unguiculata* callus (methanol extract) and methanolic leaves extract *(in vivo)* of *C. pallida* have less antioxidant activity than ethanol extract of *Crotalaria pallida.* When compared to standard (BHT) the methanolic leaves extract *(in vivo)* and methanolic callus extract *(in vitro)* of *C. pallida* shows 86.2% and 91.42% DPPH scavenging activity respectively.

The total antioxidant activity of *C. pallida* Aiton increased with increase in concentration of the extract indicating the reduction of Mo (VI) to Mo (V) and the subsequent formation of phosphomolybdate complex by donating the electron. The reducing power shown by the methanolic extract in comparison with ascorbic acid suggests that the methanolic extract is able to reduce water soluble oxidants. Antioxidant activity has been reported to go together with the development of reducing power and is a significant indicator of its potential antioxidant activity. (Yen *et al*, 1993). The methanolic leaves and callus extracts of *C. pallida* exhibited highest antioxidant activity of 94.47±0.54 and 96.5±0.51 at 50 mg/ml concentration respectively. The acetone:water (80:20) plant extract of *Indigofera colutea* was observed with antioxidant activity of 2.54±0.14 at 100 µg/ml concentration. (Bakasso *et al*, 2008). Total antioxidant capacity of ethanol extract and ethyl acetate increased with concentration dependent manner. Ethanol extract and ethyl acetate fraction of pod wall of *Cicer arietinum* L showed maximum total antioxidant capacity at 10 mg/ml concentration (Vadnere *et al*., 2012).

Hydroxyl radicals are the major active oxygen species causing lipid oxidation and enormous biological damage (Aurand *et al*, 1977). Hydroxyl radical scavenging activity of methanolic leaves extract *(in vivo)* and methanolic callus extract (in vitro) of *C. pallida* exhibited 21.72% and 24.42% at 50 mg/ml concentration with IC_{50} value of 90.18±3.57 mg/ml, 77.41±2.57 mg/ml respectively and IC_{50} value of 31.71±0.29 mg/ml for BHT (STD). The alcohol- water (1:1) extract of *Psoralea corylifolia* L. tested for hydroxyl scavenging activity showed significant scavenging activity of 87% at 20µg/ml concentration (Kiran and Raveesha, 2010). The petroleum ether, benzene, chloroform, ethyl acetate and methanol extract samples of *Indigofera tinctoria* exhibited OH scavenging activity ranging between 22.41±5.5% and 55.14±7.52% at 250µg/ml concentration (Nagarajan anusya and Sellamuthu manian, 2013). The dichloromethane: methanol (1:1, v/ v) callus extract of *Santalum album L* exhibited OH radical scavenging activity of 94.5% at 100 µg /ml concentration (Biswapriya, 2012). When compared to standard (BHT) the methanolic leaves extract *(in vivo)* and methanolic callus extract *(in vitro)* of *C. pallida* shows 35.16% and 40.96% hydroxyl scavenging activity respectively.

Superoxide anions indirectly initiated lipid oxidation as a result of superoxide and hydrogen peroxide serving as precursors of singlet oxygen and hydroxyl radicals (Okuda *et al*, 1983). Superoxide anion radical scavenging activity of methanolic leaves and callus extracts of *C. pallida* exhibited 26.8% and 43.89% at 50 mg/ml concentration with IC_{50} value of 72.58±1.55 mg/ml, 42.96±0.29 mg/ml respectively and IC_{50} value of 37.23±0.39 mg/ml for BHT (STD). Superoxide anion radicals are produced by a number of cellular reactions, including various enzyme systems such as lipoxygenases, peroxidase, NADPH oxidase and xanthine oxidase. Superoxide anion plays an important role in plant tissue and also involving in formations of other cell damaging free radicals (Blokhina *et al*., 2003). Different concentrations (1-1280µg/ml) of the methanolic extract of the roots of *C .burhia* were tested for Superoxide anion radical scavenging activity which exhibited 96.66% of scavenging activity at 1280µg/ml concentration

(Gaurav *et al*, 2010). The Superoxide scavenging activity of leaf, stem, and inflorescence callus extracts (80% methanol) of *Ocimum sanctum* at the 1.0mg/ml concentration was 87, 76, and 60%, respectively (Lukmanul hukkim *et al*., 2007). When compared to standard (BHT) the methanolic leaves and callus extracts of *C. pallida* shows 51.29% and 86.66% superoxide scavenging activity respectively.

CONCLUSION

The antioxidative data presented in this study demonstrates that *Crotalaria pallida* Aiton exhibits enzymatic and free radical scavenging activity as well as primary antioxidant activity that react with free radicals, which may limit free radical damage occurring in human body. Further studies are needed with this plant under study to, characterize and elucidate the structure of the bioactive compounds of this plant for industrial drug formulation and to purify proteins from this plant which may act as a drug for the treatment of various diseases.

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