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Full Proceeding Paper

ANTIOXIDANT ACTIVITY OF *COLDENIA PROCUMBENS* LINN. WHOLE PLANT METHANOLIC EXTRACT

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ABSTARCT

The antioxidant potential of whole plant alcoholic extract of *Coldenia procumbens* Linn. was evaluated by using DPPH Radical-Scavenging activity, ABTS radical cation scavenging activity, Ferric-reducing power (FRAP) assay, *In vitro* anti-lipid peroxidation assay (using TBARS) and Superoxide Scavenging Assay. The total phenolic content (TPC) from extract was also quantified using Folin - Ciocalteu's method with minor modifications. The alcoholic extract showed significant antioxidant potential was observed in all these assays. Total phenolic content of the extract in 1000 µg/ml was equivalent to 200 µg/ml of gallic acid. The results thus obtained suggest satisfactory antioxidant activity of whole plant alcoholic extract of *Coldenia procumbens* Linn..

INTRODUCTION

Coldenia procumbens Linn. is an annual herb; common weed widespread throughout Africa, tropical Asia and Australia at altitudes up to 750 m. In India, it is found widely in South India on waste lands, common in dry rice grounds. It belongs to family *Boraginaceae*. It is commonly referred as Creeping Coldenia, in Ayurveda it is referred as Tripakshee. This plant is widely used in traditional medicines in India, Africa, Malaysia. Pulp of fresh leaves of *Coldenia procumbens* Linn. are applied to rheumatic swellings. The dried and powdered form of the plant is applied to mature abscesses. Fresh leaves are used as a poultice on mature abscesses. It is used for the suppuration of boils, fever, piles and scorpion sting. Plant extract also used in treatment of piles, Leucorrhoea, Menorrhagia diabetes, anti-arthritic and hypotensive.

Pharmacological screening of an methanolicextract of whole *Coldenia procumbens* Linn. plants showed the central nervous system depression in mice and the pentobarbital-induced sleeping time prolongation in rats. Acetone, ethanol and water extracts of dried aerial parts have shown weak angiotensin-converting enzyme inhibition *In vitro* [1]. The aqueous extract of the *Coldenia procumbens* Linn. had shown antibacterial activity [2, 3].

Coldenia procumbens Linn. have the glycosides, phytosterols, proteins, amino acids, fixed oils flavonoids, gums and mucilage as chief constituents. Alkaloids and tannins are higher in alcoholic extract than in water extract. Reducing sugars and phenols are higher in water extract than in alcoholic extract. Saponins and fixed oils and fats are present only in water and alcoholic extracts respectively.

The aim of present study was to screen methanolicextract of *Coldenia procumbens* Linn. whole plant for its antioxidant activity.

MATERIAL AND METHODS

Collection of plant materials and plant extraction

The whole plant of Coldenia procumbens Linn. was collected from Pudukkottai district, Tamilnadu, India. It was authenticated by Dr.Sasikala Ethirajulu, Siddha Central Research Institute. A voucher specimen (ACC. No. 7311) has been deposited in the Institute. Shade dried and coarsely powdered plant (1 kg) was extracted successively with chloroform and methanol in a Soxhlet apparatus. The extracts were filtered through Whatman No.1 filter paper and distilled on a water bath to get a syrupy mass. The extracts were then dried in vacuum (yield 23 and 20 gram respectively). The dissolved extract was then lyophilised using lyophilizer (Labconco freezone 4.5) at -50^{0}C and 0.020mBar pressure for three days. Later, the lyophilised material (5mg/ml) was used for the preparation of the drug ranging from concentration of 1000µg/ml to 10µg/ml. This was further used to assess the antioxidant activity.

Determination of antioxidant by various anti-oxidant assays

Reducing power / FRAP (Ferric reducing antioxidant potential assay)

The reducing power of plant extracts was determined according to the method of Oyaizu (1986) [4]. About 200 mg of plant extract in 1ml of distilled water was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide [K3Fe(CN)6] (2.5 ml, 1%). The mixture was incubated at 50OC for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 g for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl3 (0.5 ml, 0.1%) and the absorbance was measured at 700 nm. BHT was used as the reference material. All the tests were performed in triplicate and the graph were plotted with the average of three observations. The absorbance obtained was converted to BHT equivalent in mg per gm of dry material (BHT/g) using BHT standard curve.

Inhibition of DPPH radical

The free radical scavenging activity of plant extracts was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH) using the method of Blois (1958) [5] and Gomez-Alonso *et al*., (2003) [6]. 0.1 mM solution of DPPH in methanol was prepared and 1ml of this solution was added to 3 ml of various concentration of plant extracts and the reference compound (50, 100, 150, 200 and 250 µg). After 30 min, absorbance was measured at 517 nm. BHA was used as the reference material. The percentage of inhibition was calculated by comparing the absorbance values of the control and test samples.

ABTS radical cation decolorisation assay

In this assay, the oxidant is generated by persulfate oxidation of 2, 2,-azinobis (3-ethylbenzoline-6-sulfonic acid)-($ABTS^{2-}$) as described by Re *et al*., (1999) [7]. ABTS radical cation ($ABTS^{+}$) are produced by reacting ABTS solution (7mM) with 2.45 mM ammonium persulphate and the mixture was allowed to stand in dark at room temperature for 12-16 hrs before use. After 16 hrs, this solution was diluted with ethanol until the absorbance reaches 0.7 ± 0.02 at 734 nm. For the study, different concentration of plant extract was added to 0.3ml of ABTS solution and the final volume was made up with ethanol to make 1ml. The absorbance was read at 745nm and the percentage inhibition calculated.

Total Phenolic Content (TPC)

Total phenolic content (TPC) from extracts were quantified using Folin - Ciocalteu's method adapted to 96-well microtitre plate with minor modifications (Slinkard and Singleton, 1977) [8]. 100 µl of 1:4 diluted Folin - Ciocalteu's phenol reagent, 2N (Sigma - Aldrich) in distilled water was added to 20 µl of serially diluted (10 – 1000 µg/ ml) lyophilized plant extracts and standard gallic acid dissolved in distilled water. After 5 min incubation at room temperature, 80 µl of sodium carbonate (75 g/L) was added to each well. 96 well plate was slightly shaken and incubated for 30 min at room temperature in the darkness. The absorbance was measured at 735 nm using ELISA plate reader. The assay was repeated thrice and Total phenolic contents (average of three) were expressed as BHT equivalent per gram of lyophilized extract.

Superoxide Scavenging Assay

Measurement of superoxide anion scavenging activity of plant extract was performed based on the method described by Nishimiki *et al.,* (1972) [9] with slight modifications. About 1ml of Nitroblue Tetrazolium (NBT) solution containing 156µM NBT dissolved in 1.0 ml of phosphate buffer (100mM, pH 7.4) and 1ml of NADH solution containing 468 µM of NADH which is dissolved in 1ml of phosphate buffer (100 mM, pH 7.4) with 0.1 ml of various concentrations of plant extracts and the reference compounds (10, 20, 30, 40 µg) were mixed and the reaction was started by adding 100 µl of Phenazine methosulphate (PMS) solution containing 60 µM of PMS 100 µl of phosphate buffer (100 mM, pH 7.4). The reaction mixture was incubated at 25OC for 5 min and the absorbance at 560nm was measured against the control samples. BHT was used as the reference compounds. All the tests were performed in triplicate and the results were arranged. The percentage inhibition was calculated by comparing the results of control and test samples.

***In vitro* anti-lipid peroxidation assay using TBARS**

Thiobarbituric acid reactive species (TBARS) assay was performed as described by Aazza *et. al.*, 2011 [10]. Egg yolk homogenates were used as a lipid-rich medium obtained as described elsewhere [11]. Briefly, 100 µl of egg yolk [(10% w/v) in KCl (1.15%)] and 50 µl of extract or standard vitamin E in ethanol (10 - 1000 µg/ ml) were added. Then, 300 µl of 20% acetic acid (pH 3.5) and 300 µl of 0.8% (w/v) TBA in 1.1% (w/v) sodium dodecyl sulphate (SDS) were added. The resulting mixture was vortexed and heated at 95°C for 60 min. After cooling, at room temperature, 750 µL of butan-1-ol was added to each tube; the contents of the tubes were stirred and centrifuged at 3000 rpm for 10 min. The upper organic layer was transferred to 96-well microtitre plate and absorbance was measured at 532 nm using a ELISA plate reader. All of the values were based on the percentage antioxidant index (AI%), whereby the control was completely peroxidized and each sample demonstrated a degree of change; the percentage inhibition was calculated using the formula (1- T/C) × 100, where C is the absorbance value of the fully oxidized control and T is the absorbance of the test sample. The antioxidant capacity was determined from three replicates. The percentage antioxidant index was plotted against the concentrations of samples and IC50 values were determined. Same amount of deionized water was used as the control.

RESULTS

Ferric-reducing power (FRAP) assay

The *C. procumbens* extract showed to have 1000 µg equivalent to BHT/mg of sample (Figure 1).

Inhibition of DPPH radical

The potential decrease in the concentration of DPPH radical due to the scavenging ability of BHA standard and *Coldenia* extract showed significant DPPH scavenging activity: at 50 µg/ml concentration 89.19% & 53.60% inhibition was observed for BHA and *C. procumbens* extract respectively (Figure 2).

The IC_{50} of DPPH was seen at concentration of 45 µg/ml for *C. procumbens* extract.

ABTS radical cation scavenging activity

The potential decrease in the concentration of ABTS radical was due to the scavenging ability of Gallic acid standard and *Coldenia* extract showed significant ABTS scavenging activity: at 500 µg/ml concentration 90.72% & 90.18% inhibition was observed for Gallic acid standard and *C. procumbens* extract respectively (Figure 3).

IC_{50} of ABTS was seen at concentration of 200 µg/ml for *C. procumbens* extract (Figure 8).

Total Phenolic Content

The *C. procumbens* 100 µg/ml extract contains 12.0943 µg BHT equivalent of total phenol (Figure 4).

Superoxide Scavenging Assay

The potential increase in the super oxide scavenging ability of BHT standard and *Coldenia* extract showed significant superoxide scavenging activity: at 500 µg/ml concentration 92.91% & 72.77% inhibition was observed for BHT standard and *C. procumbens* extract respectively (Figure 5).

The IC_{50} of Superoxide Scavenging assay was seen at concentration of 70 µg/ml for *C. procumbens* extract.

***In vitro* anti-lipid peroxidation assay using TBARS**

The potential anti-lipid peroxidation ability of Vitamin E standard and *Coldenia* extract was observed. Thiobarbituric acid reactive substances scavenging activity for *Coldenia* extract at 500 µg/ml concentration was 51.82% & 47.17% inhibition was observed for Vitamin E.

The IC_{50} TBARS inhibition was seen at concentration of 1000 µg/ml for *C. procumbens* extract (Figure 6).

DISCUSSION

Free radicals are chemical entities that can co-exist with one or more unpaired electrons. The generation of free radicals has extensive tissue damaging effect. Lipids, proteins, nucleic acids are all susceptible to attack by free radicals [12]. Oxidative stress, in which large quantities of reactive oxygen species (ROS) like hydrogen peroxide, superoxide (O_2^-), hydrogen radical (OH^-), singlet oxygen and nitrogen species are generated, is one of the earliest responses to stress. These ROS have a role in disease, inflammation and aging in animals. The diseases such as cataract, liver cirrhosis, ischemia/reperfusion injury, Parkinson disease, Alzheimer type dementia, AIDS, arthritis, cancer, cardiovascular dysfunction, diabetes, retinopathy and rheumatism are the direct consequence of oxidative injuries. The antioxidative system by scavenging the free radicals protects the body tissues against ROs-induces oxidative damage. Herbal drugs contain radical scavengers and so are gaining importance in the prevention and treatment of these diseases. Phenolic compounds and flavonoids are the major constituents in plants possessing antioxidant and free radical scavenging activity as they are effective hydrogen donors [13].

The FRAP assay takes advantage of electron-transfer reactions. Here in a ferric salt, Fe (III) $(TPTZ)_2$ Cl_3 (TPTZ) 2, 4, 6-tripyridyls-triazine), is used as an oxidant. The change in absorbance is therefore, directly related to the combined or "total" reducing power of the electrondonating antioxidants present in the reaction mixture. Total antioxidant activity is measured by ferric reducing antioxidant power (FRAP) assay of Benzie and Strain (1999) [14]. *C. procumbens* extract showed FRAP potential almost equivalent to the standard (BHT) which is a remarkable of it. The free radical scavenging capacity of alcoholic extract of *C procumbens* was tested by its ability to bleach the stable DPPH radical that was observed as a decrease in absorbance of DPPH radicals at 517nm. DPPH is a stable free radical at room temperature and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [15]. The significant decrease in the concentration of the DPPH radical was due to the scavenging activity of *C. procumbens* extract.

The ABTS assay is based on the inhibition of the absorbance of the radical cation $ABTS^{+}$that has a long wavelength absorption spectrum. The results imply that the *Coldenia* extract scavenged the $ABTS^{+}$radicals effectively.

The hydroxyl groups on phenols provide them with a free radical scavenging activity. They contribute directly to the antioxidant activity [16]. In the *C. procumbens* 100 µg/ml extract, 12.0943 µg BHT equivalent of total phenol was detected. The results indicate strong association between antioxidative activities and phenolic compounds (r^2= 0.9998), suggesting that the phenolic compounds are responsible for the antioxidative activities of *C. Procumbens*.

Superoxide radical is known to be a very harmful species to cellular components as a precursor of more reactive oxygen species (ROS). Superoxides are produced from molecular oxygen due to oxidative enzymes of body as well as via non-enzymatic reaction such as autoxidation by catecholamines. In the present study, superoxide radical reduced NBT to a blue colored formazan that was measured at 560 nm. The superoxide scavenging effect of the ethanol extract of *C. procumbens* and the standard drug (BHT) on the PMS/NADH-NBT system was assessed. The decrease of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture.

Lipid peroxidation was followed through the method known as TBARS, which measures the malonaldehyde, a secondary product of lipid oxidation using egg yolk as lipidic substrate; being temperature the chosen condition for accelerating the peroxidation process. The results obtained indicate that the *C. procumbens* has satisfactory Thiobarbituric acid reactive substances scavenging activity (TBARS).

So at last it can be concluded based on the various assay results obtained that *Coldenia procumbens* Linn. whole plant alcoholic extract has a good antioxidant activity and its importance is once again highlighted for reducing the damaging oxidative stresses occurring routinely in human body.

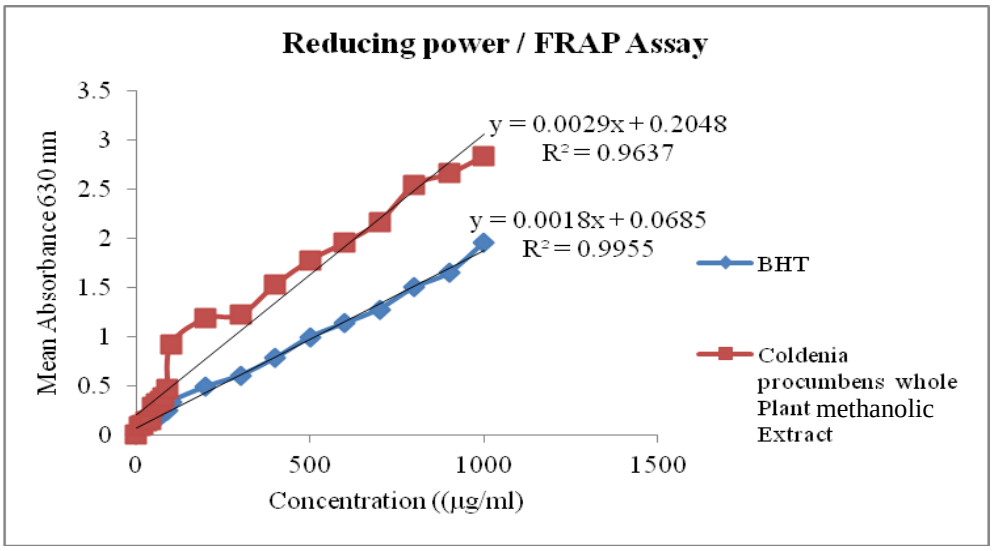

Fig. 1: Ferric reducing activity of *Coldenia procumbens* Linn. whole plant methanolic extract and BHT. Increase in absorbance of the reaction mixture indicates the increase in reducing power.

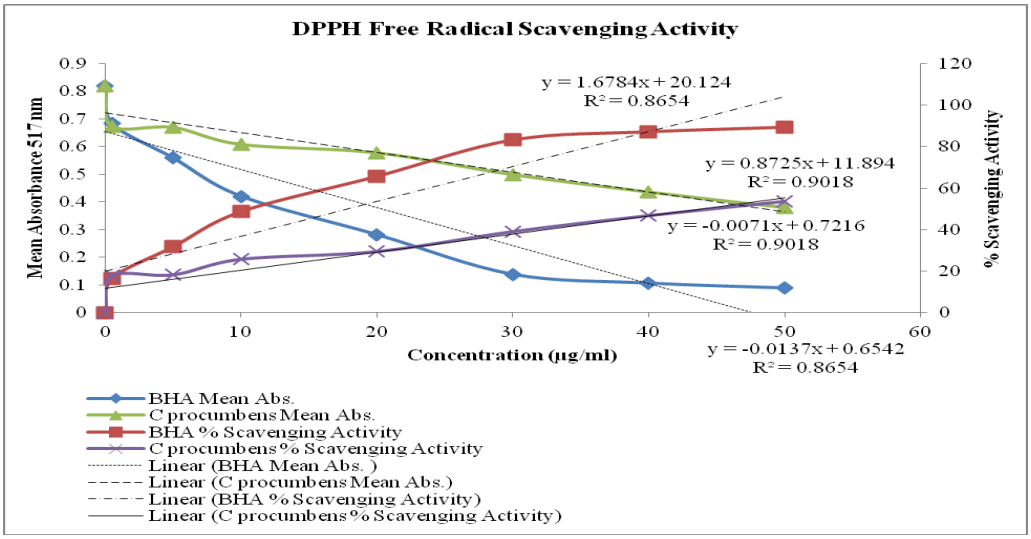

Fig. 2: DPPH radical-scavenging activity of *Coldenia procumbens* Linn. whole plant methanolic extract and BHA. Decrease in mean absorbance with increase in concentration of the reaction mixture indicates the increase in oxidising power and % Radical Scavenging.

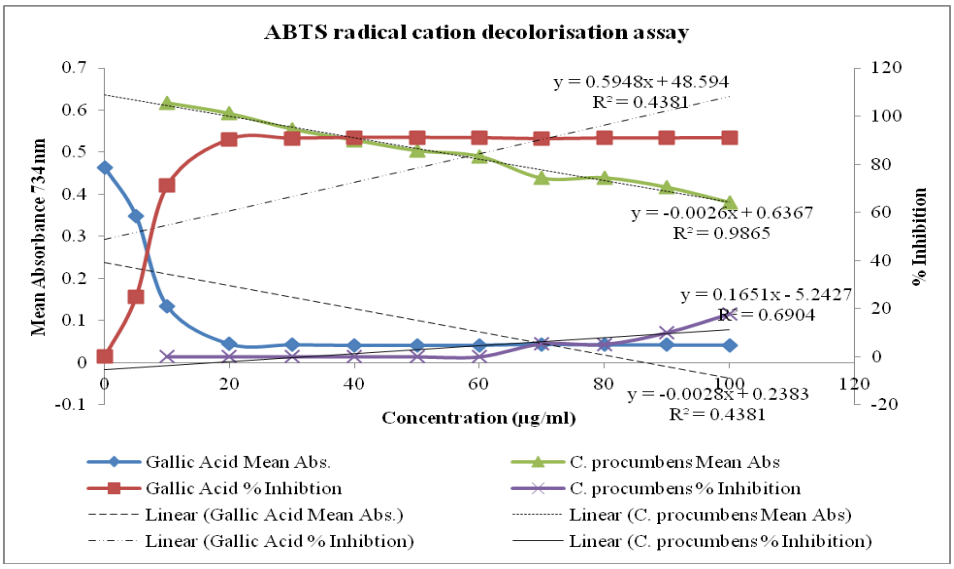

Fig. 3: ABTS radical-scavenging activity of *Coldenia procumbens* Linn. whole plant methanolic extract and Gallic acid. Decrease in mean absorbance with increase in concentration of the reaction mixture indicates the increase in oxidising power and % Inhibition.

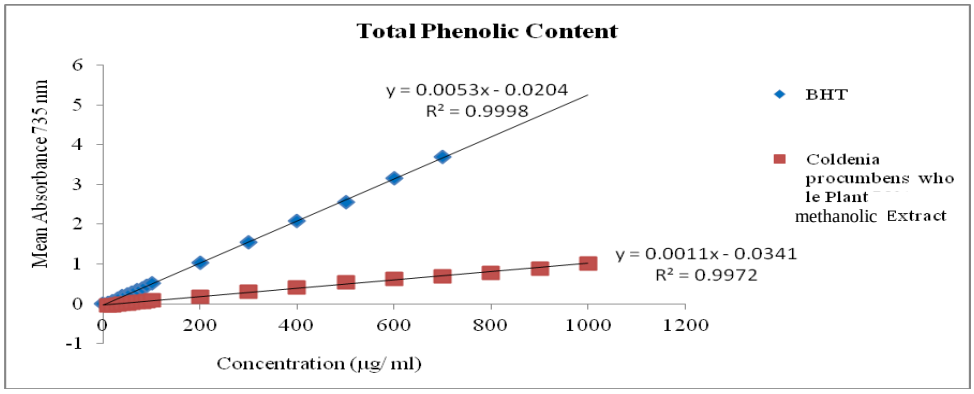

Fig. 4: Total Phenolic Content (TPC) assay

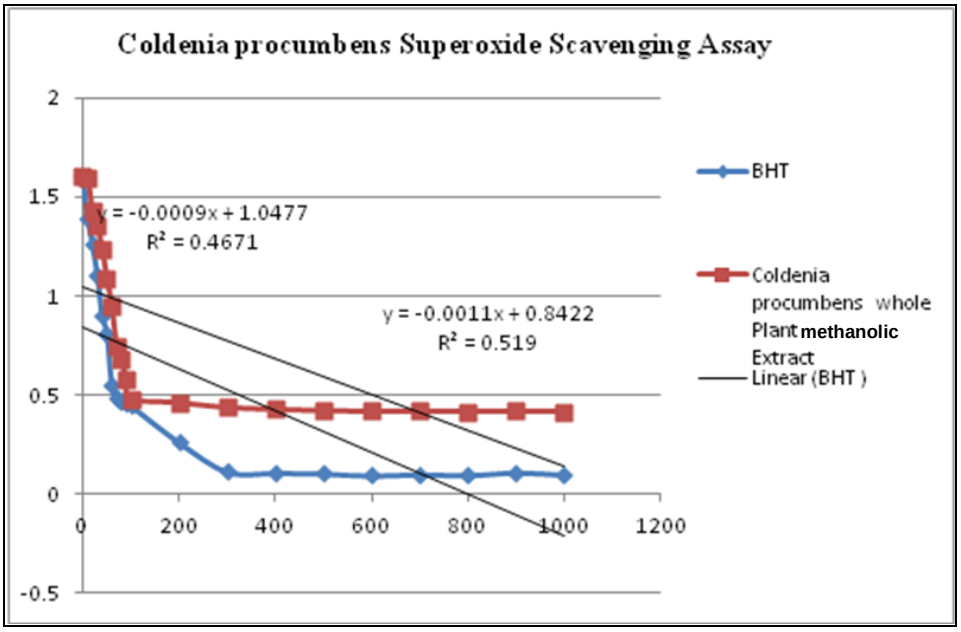

Fig. 5: Superoxide Scavenging assay

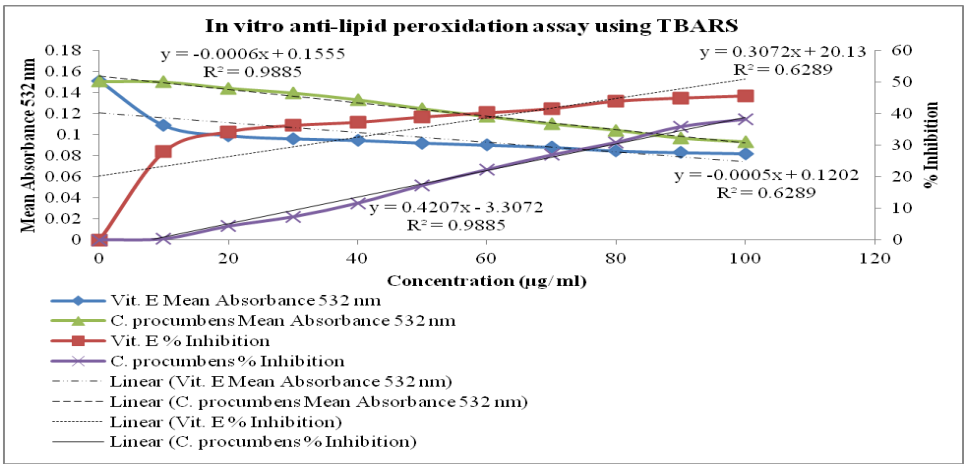

Fig. 6: *In vitro* anti-lipid peroxidation assay using TBARS assay of *Coldenia procumbens* Linn. whole plant methanolic extract and vitamin E.

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