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DETERMINATION OF *IN VITRO* ANTIOXIDANT EFFICACY OF *CISSUS VITIGINEA* LEAF EXTRACT

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Keywords:

Antioxidant activity, *Cissus vitiginea* leaf, Radical scavenging, Reactive Oxygen species

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ABSTRACT

The methanolic extract *Cissus vitiginea* leaf was screened for *in vitro* antioxidant activity by oxygen radical scavenging such as DPPH, total antioxidant assay, superoxide metal chelation and iron reducing power activity at different concentrations. Throughout the studies leaves extract showed marked antioxidant activity. The antioxidant activity of the leaves extract may be due to the phytochemical present in it. The antioxidant activity was found to be concentration dependent and may be attributed to the presence of bioflavonoid content in the leaves of *Cissus vitiginea.* Overall, the plant extract is a source of natural antioxidants which might be helpful in preventing the progress of various oxidative stress mediated diseases including aging.

INTRODUCTION

About 5% or more of the inhaled oxygen (O_2) is converted to reactive oxygen species such as O_2, H_2O_2, and OH. Limited production of reactive oxygen species is not harmful. But overproduction of free radicals or reactive oxygen species (ROS) contributes to oxidative stress, which leads to damage of proteins, DNA and lipids that is associated with chronic degenerative diseases, including cancer, coronary artery diseases, hypertension and diabetes etc. [1, 2]. The human body has an elaborate antioxidant defence system. Antioxidants are manufactured within the body and can also be extracted from the food humans eat such as fruits, vegetables, seeds, nuts, meats and oil [3]. Antioxidants can act by scavenging reactive oxygen species, inhibiting their formation, binding transition metal ions and preventing formation of OH and/or decomposition of lipid hydroperixides, which could lead to the repairing of damages [4]. Antioxidants play an important role in providing protection to humans against infection and degenerative diseases. Antioxidants are classified into two major categories, natural and synthetic. Several synthetic antioxidants, such as butylated hydroxyl anisole and hydroxyl toluene, are commercially available and are used in 50-200 ppm in foods and have many side effects such as mutagenesis carcinogenic in human beings [5, 6].

Natural antioxidants are safe and also bioactive.Among the various natural products, phenolic compounds are natural antioxidants that have the character of quenching oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radical. In addition, these compounds have anti-inflammatory, anti-carcinogenic and anti-atherosclerotic activities [7, 8]. Therefore, recently, wide investigations have been done for identification of plants with antioxidant activity that may be used for treatment of various diseases in human [9]. The aim of this study was designed for the evaluation of *in vitro* antioxidant activity of *Cissus vitiginea* leaf L. [Family VITACEAE] through DPPH, superoxide anion scavenging and metal chelator (iron chelator and iron reducing power) and in order to identify new sources of natural antioxidants.

MATERIALS AND METHODS

Chemicals:

Nitro blue tetrazolium (NBT), ethylene diamine tetra acetic acid (EDTA), sodium nitroprusside (SNP), trichloro acetic acid (TCA), thio barbituric acid (TBA), potassium hexa cyano ferrate [$K_3Fe(CN)_6$], and L-ascorbic acid were purchased from Sisco Research Laboratories Pvt. Ltd., India. All other chemicals and solvents used were of analytical grade available commercially.

Plant materials:

The *Cissus vitiginea* leaves were collected in January 2015 from Tamil University, Thanjavur District, Tamil Nadu, India from a single herb. The leaves were identified and authenticated by Dr. S. John Britto, The Director, the Rabiant Herbarium and centre for molecular systematics, St. Joseph's college Trichy-Tamil Nadu. India. A Voucher specimen has been deposited at the Rabinat Herbarium, St. Josephs College, Thiruchirappalli, Tamil nadu, India.

Preparation of alcoholic extract:

The collected *Cissus vitiginea* leaves were washed several times with distilled water to remove the traces of impurities from the leaves. The plant was dried at room temperature and coarsely powdered. The powder was extracted with 70% methanol for 48 hours. A semi solid extract was obtained after complete elimination of alcohol under reduced pressure. The extract was stored in desiccator until used. The extract contained both polar and non-polar phytocomponents of the plant material used.

In vitro antioxidant activity:

DPPH assay:

The scavenging ability of the natural antioxidants of the plant extract towards the stable free radical DPPH was measured by the method of [10]. Briefly, a 2 ml aliquot of DPPH methanol solution (25µg/ml) was added to 0.5 ml sample solution at different concentrations. The mixture was shaken vigorously and allowed to stand at room temperature in the dark for 30 min. Then the absorbance was measured at 517nm in a spectrophotometer. L-Ascorbic acid was used as the standard.

$$\text{Radical scavenging activity (\%)} = 100 - \left(\frac{A_C - A_S}{A_C} \right) \times 100$$

Where $A_{C\ =}$ control is the absorbance of the control and $A_{S\ =}$ sample is the absorbance of reaction mixture (in the presence of sample). All tests were run in triplicates (n = 3), and the average values were calculated.

Determination of total antioxidant capacity:

The antioxidant activity of the extracts was evaluated by the phosphomolybdenum method according to the procedure of [11]. The assay is based on the reduction of Mo(VI)–Mo(V) by the

extract and subsequent formation of a green phosphate/Mo(V) complex at acid pH. 0.3 ml extract was combined with 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer against blank after cooling to room temperature. Methanol (0.3 ml) in the place of extract is used as the blank. The antioxidant activity is expressed as the number of equivalents of ascorbic acid.

Superoxide anion scavenging activity assay:

The scavenging activity of the *Cissus vitiginea* towards superoxide anion radicals was measured by the method of [12]. Superoxide anions were generated in a non-enzymatidc phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system through the reaction of PMS, NADH, and oxygen. It was assayed by the reduction of nitroblue tetrazolium (NBT). In these experiments the superoxide anion was generated in 3 ml of Tris-HCl buffer (100mM, pH 7.4) containing 0.75 ml of NBT (300 μM) solution, 0.75 ml of NADH (936 μM) solution and 0.3 ml of different concentrations of the extract. The reaction was initiated by adding 0.75 ml of PMS (120 μM) to the mixture. After 5 min of incubation at room temperature, the absorbance at 560 nm was measured in spectrophotometer. The superoxide anion scavenging activity was calculated according to the following equation:

$$\% \text{ Inhibition} = \frac{A_o - A_1}{A_0} \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Fe^{2+} chelating activity assay:

The chelating activity of the extracts for ferrous ions Fe^{2+} was measured according to the method of [13]. To 0.5 ml of extract, 1.6 ml of deionized water and 0.05 ml of $FeCl_2$ (2 mM) was added. After 30 s, 0.1 ml ferrozine (5 mM) was added. Ferrozine reacted with the divalent iron to form stable magenta complex species that were very soluble in water. After 10 min at room temperature, the absorbance of the Fe^{2+}–Ferrozine complex was measured at 562 nm. The chelating activity of the extract for Fe^{2+} was calculated as

$$\% \text{ Inhibition} = \frac{A_o - A_1}{A_0} \times 100$$

Where A_0 was the absorbance of the control (blank, without extract) and A_1 was the absorbance in the presence of the extract.

Reducing power assay:

The Fe^{3+} reducing power of the extract was determined by the method of [14] with slight modifications. The extract (0.75 ml) at various concentrations was mixed with 0.75 ml of phosphate buffer (0.2 M, pH 6.6) and 0.75 ml of potassium hexacyanoferrate [$K_3Fe(CN)_6$] (1%, w/v), followed by incubating at 50°C in a water bath for 20 min. The reaction was stopped by adding 0.75 ml of trichloroacetic acid (TCA) solution (10%) and then centrifuged at 3000 r/min for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1 ml of ferric chloride ($FeCl_3$) solution (0.1%, w/v) for 10 min. The absorbance at 700 nm was measured as the reducing power. Higher absorbance of the reaction mixture indicated greater reducing power.

Statistical analysis:

Tests were carried out in triplicate for 3–5 separate experiments. The amount of extract needed to inhibit free radicals concentration by 50%, IC_{50}, was graphically estimated using a nonlinear regression algorithm.

RESULTS AND DISCUSSION

DPPH assay:

DPPH radical scavenging activity of plant extract of *Cissus vitiginea* leaf and standard as ascorbic acid are presented in Fig 1 and Table 1. The DPPH radical was widely used to evaluate the free-radical scavenging capacity of antioxidants [15]. Recently, the use of the DPPH$^{\bullet}$ reaction has been widely diffused among food technologists and researchers, for the evaluation of free radical scavenging activity on extracts from plant, food material or on single compounds. In the DPPH assay, the antioxidant was able to reduce the stable radical DPPH to the yellow colored 1, 1-diphenyl-1, 2-picryl hydrazine. The molecule of 2, 2-diphenyl-1-picryl hydrazine is characterised as a stable free radical by virtue of the delocalization of the spare electron over the molecule as a whole. The proton transfer reaction of the DPPH$^{\bullet}$ free radical by a scavenger (A-H) causes a decrease in absorbance at 517 nm, which can be followed by a common

spectrophotometer set in the visible region. The effect of antioxidants on $DPPH^{\bullet}$ is thought to be due to their hydrogen donating ability [16]. The half inhibition concentration (IC_{50}) of plant extract and ascorbic acid were 45.12µg ml^{-1} and 34.91 µg ml^{-1} respectively**.** The plant extract exhibited a significant dose dependent inhibition of DPPH activity. The potential of L-ascorbic acid to scavenge DPPH radical is directly proportional to the concentration. The DPPH assay activity is near to standard as ascorbic acid.

TABLE 1: % OF DPPH RADICAL SCAVENGING ACTIVITY OF MEHANOLIC EXTRACT OF *CISSUS VITIGINEA* AT DIFFERENT CONCENTRATIONS

Parameters	20 (µg/ml)	40 (µg/ml)	60 (µg/ml)	80 (µg/ml)	IC_{50} (µg/ml)
Cissus vitiginea	23.63±0.95	38.18±1.27	65.45±3.18	82.27±5.41	45.12
Ascorbic acid	25.6±2.04	61.26±4.90	88.98±7.11	99.34±7.94	34.91

Values were expressed as Mean ± SD for triplicates

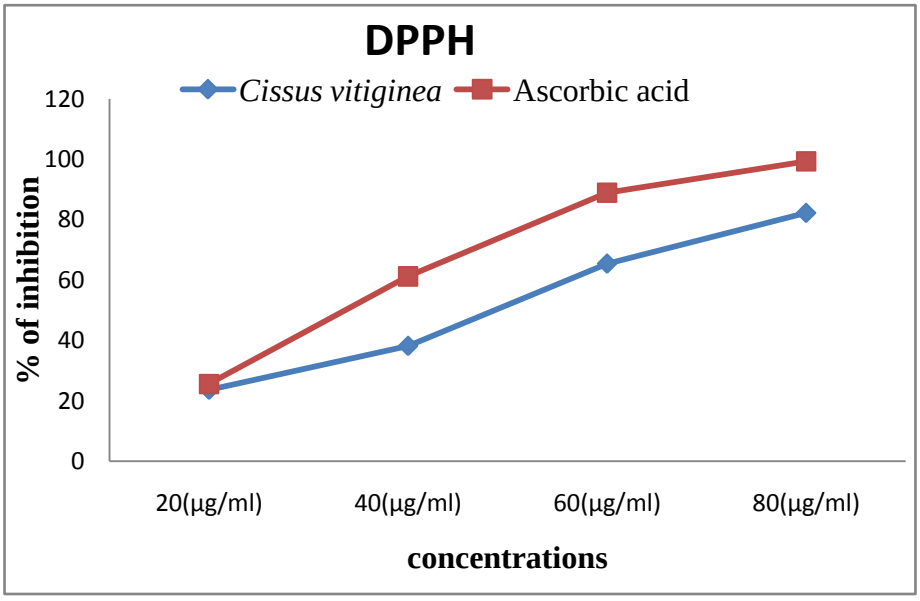

FIG 1: % OF DPPH RADICAL SCAVENGING ACTIVITY OF MEHANOLIC EXTRACT OF *CISSUS VITIGINEA* AT DIFFERENT CONCENTRATIONS

Total antioxidant activity:

The yield of the ethanol extract of the plant extract and its total antioxidant capacity are given in Fig. 2 and Table 2. Total antioxidant capacity of *Cissus vitiginea* leaf is expressed as the number

of equivalents of ascorbic acid. The phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/ Mo (V) complex with a maximal absorption at 695 nm. The assay is successfully used to quantify vitamin E in seeds and, being simple and independent of other antioxidant measurements commonly employed, it was decided to extend its application to plant extract [11]. Moreover, it is a quantitative one, since the antioxidant activity is expressed as the number of equivalents of ascorbic acid. The study reveals that the antioxidant activity of the extract is in the increasing trend with the increasing concentration of the plant extract. The half inhibition concentration (IC_{50}) of plant extract and ascorbic acid were 57.10µg ml^{-1} and 42.41 µg ml^{-1} respectively**.**

TABLE 2: % OF TOTAL ANTIOXIDANT ACTIVITY OF MEHANOLIC EXTRACT OF *CISSUS VITIGINEA AT* DIFFERENT CONCENTRATIONS

Parameters	**20 (µg/ml)**	**40 (µg/ml)**	**60 (µg/ml)**	**80 (µg/ml)**	**IC_{50} (µg/ml)**
Cissus vitiginea	16.50±6.13	25.00±5.25	50.00±3.50	71.38±0.41	57.10
Ascorbic acid	22.35± 1.80	51.23± 4.09	72.54± 5.80	86.35± 6.91	42.41

Values were expressed as Mean ± SD for triplicates

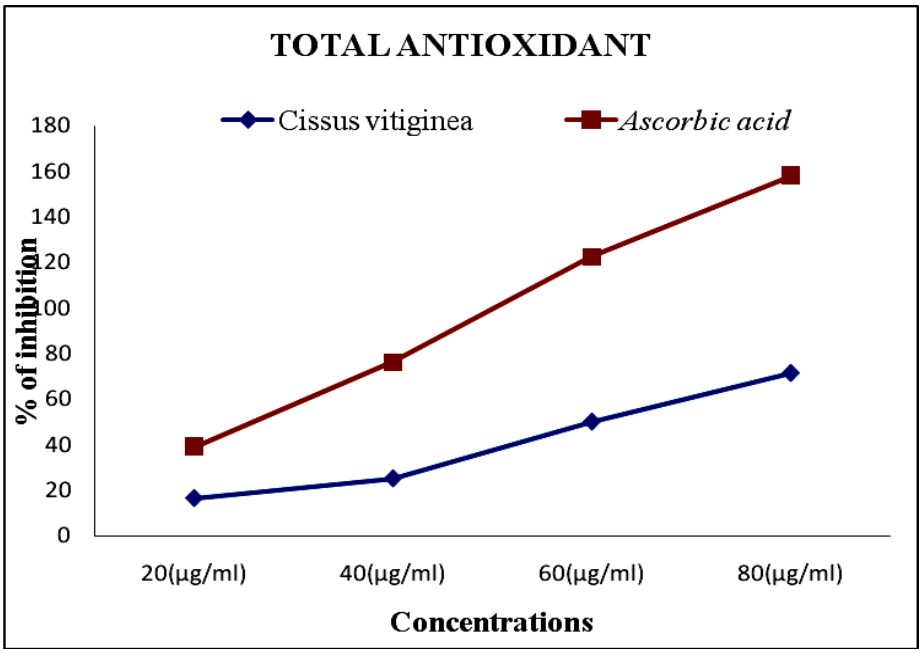

FIG 2: % OF TOTAL ANTIOXIDANT ACTIVITY OF MEHANOLIC EXTRACT OF *CISSUS VITIGINEA AT* DIFFERENT CONCENTRATIONS

Superoxide anion radical scavenging activity:

Superoxide is biologically important since it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals, is very harmful to the cellular components in a biological system [17]. The superoxide anion radical scavenging activities of the extract from *Cissus vitiginea* leaf assayed by the PMS-NADH system was shown in Fig 3and Table 3. The superoxide scavenging activity of *Cissus vitiginea* leaf was increased markedly with the increase of concentrations. The half inhibition concentration (IC_{50}) of *Cissus vitiginea* leaf was 59.61µg ml^{-1} and ascorbic acid were 31.62µg ml^{-1} respectively**.** These results suggested that *Cissus vitiginea* leaf had notably superior superoxide radical scavenging effects.

TABLE 3: % OF SUPEROXIDE RADICAL SCAVENGING ACTIVITY OF MEHANOLIC EXTRACT OF *CISSUS VITIGINEA* AT DIFFERENT CONCENTRATIONS

Parameters	20 (µg/ml)	40 (µg/ml)	60 (µg/ml)	80 (µg/ml)	IC_{50} (µg/ml)
Cissus vitiginea	17.86±1.25	39.29±2.75	65.75±6.00	82.58±2.00	59.61
Ascorbic acid	31.25 ± 2.50	64.23 ± 5.13	89.54 ± 7.16	98.51 ± 7.88	31.62

Values were expressed as Mean ± SD for triplicates

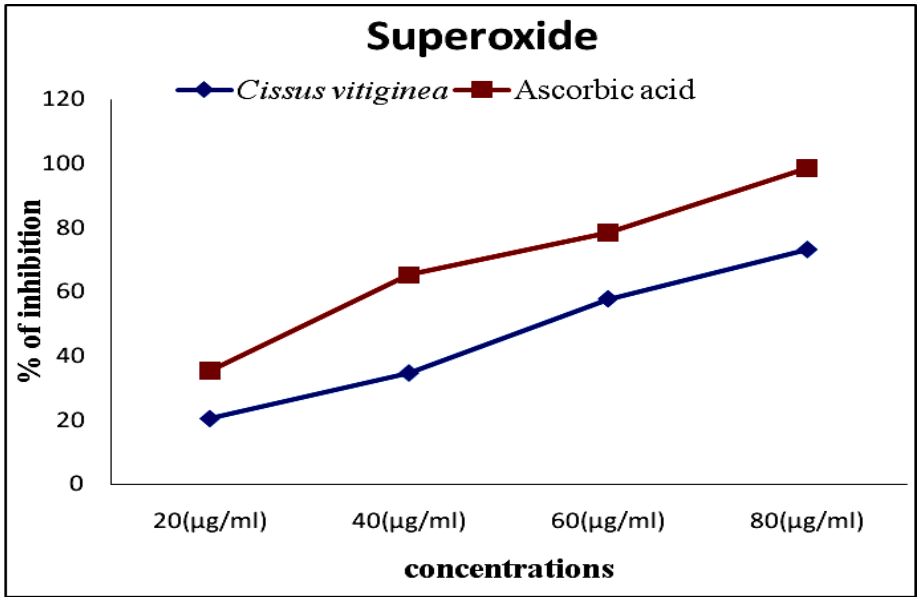

FIG 3: % OF SUPEROXIDE RADICAL SCAVENGING ACTIVITY OF MEHANOLIC EXTRACT OF *CISSUS VITIGINEA* AT DIFFERENT CONCENTRATIONS

The ferrous ion chelating activity:

Ferrozine can make complexes with ferrous ions. In the presence of chelating agents, complex (red colored) formation is interrupted and as a result, the red color of the complex is decreased. Thus, the chelating effect of the coexisting chelator can be determined by measuring the rate of color reduction. The formation of the ferrozine– Fe^{2+} complex is interrupted in the presence of aqueous extract of *Cissus vitiginea* leaf , indicating that have chelating activity with an IC_{50} of 64.70µg ml^{-1} and ascorbic acid was 30.96µg ml^{-1} respectively Fig. 4 and Table 4 . Ferrous iron can initiate lipid peroxidation by the Fenton reaction as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals [18]. Metal chelating activity can contribute in reducing the concentration of the catalyzing transition metal in lipid peroxidation. Furthermore, chelating agents that form s bonds with a metal are effective as secondary antioxidants because they reduce the redox potential, and thereby stabilize the oxidized form of the metal ion [19]. Thus, *Cissus vitiginea* leaves demonstrate a marked capacity for iron binding, suggesting their ability as a peroxidation protector that relates to the iron binding capacity.

TABLE 4: % OF IRON CHELATING ACTIVITY OF MEHANOLIC EXTRACT OF *CISSUS VITIGINEA* AT DIFFERENT CONCENTRATIONS

Parameters	20 (µg/ml)	40 (µg/ml)	60 (µg/ml)	80 (µg/ml)	IC_{50}(µg/ml)
Cissus vitiginea	20.41±2.69	34.62±2.42	57.70±4.03	73.08±5.11	64.70
Ascorbic acid	35.23 ± 2.81	65.21 ± 5.28	78.51± 6.28	98.65 ± 7.89	30.96

Values were expressed as Mean ± SD for triplicates

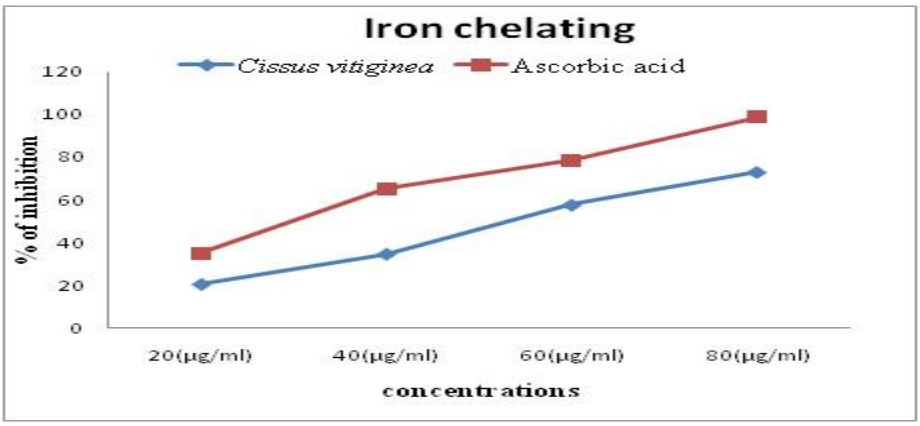

FIG 4: % OF IRON CHELATING ACTIVITY OF MEHANOLIC EXTRACT OF *CISSUS VITIGINEA* AT DIFFERENT CONCENTRATIONS

Reducing power activity:

For the measurements of the reducing ability, the Fe^{3+}–Fe^{2+} transformation was investigated in the presence of *Cissus vitiginea* leaf. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. However, the activity of antioxidants has been assigned to various mechanisms such as prevention of chain initiation, binding of transition-metal ion catalysts, decomposition of peroxides, and prevention of continued hydrogen abstraction, reductive capacity and radical scavenging [20]. Fig. 5 and Table 5 depicts the reductive effect of *Cissus vitiginea* leaf. Similar to the antioxidant activity, the reducing power of *Cissus vitiginea* leaf increased with increasing dosage. All the doses showed significantly higher activities than the control exhibited greater reducing power, indicating that *Cissus vitiginea* leaf consist of hydrophilic polyphenolic compounds that cause the greater reducing power.

TABLE 5: REDUCING POWER ASSAY OF MEHANOLIC EXTRACT OF *CISSUS VITIGINEA* AT DIFFERENT CONCENTRATIONS

Parameters	20 (µg/ml)	40 (µg/ml)	60 (µg/ml)	80 (µg/ml)
Cissus vitiginea	0.20±0.014	0.55±0.039	0.77±0.054	0.87±0.061
Ascorbic acid	0.41± 0.03	0.71 ± 0.05	0.89± 0.07	0.98 ± 0.08

Values were expressed as Mean ± SD (Optical density) for triplicates

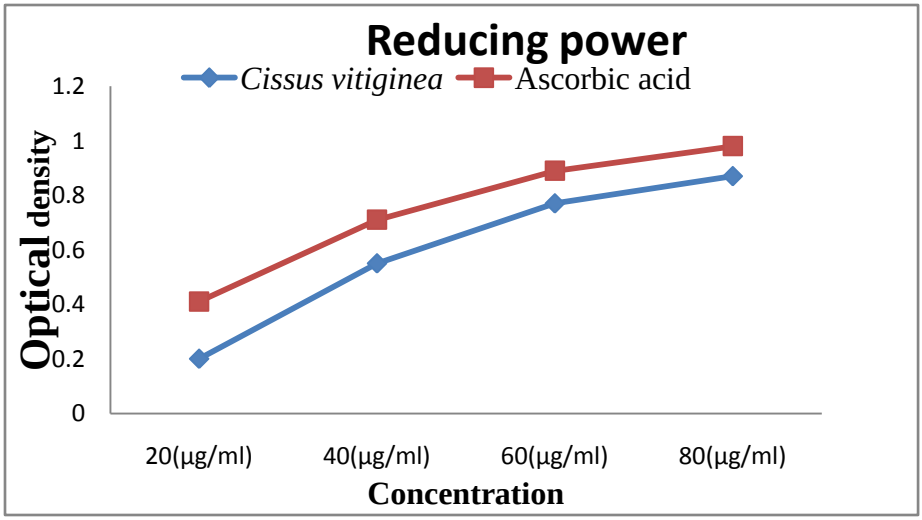

FIG 5: REDUCING POWER ASSAY OF MEHANOLIC EXTRACT OF *CISSUS VITIGINEA* AT DIFFERENT CONCENTRATIONS

CONCLUSION

The results of the present study showed that the extract of *Cissus vitiginea* leaf leaves extract which contains of flavonoids and polyohenols. These phytochemicals are exhibited the greatest antioxidant activity DPPH, superoxide anion scavenging and metal chelator (iron chelator and iron reducing power) which participate in various pathophysiology of diseases including cancer, diabetic, ageing etc. This work has gathered experimental evidence on the *Cissus vitiginea* leaf as natural antioxidant for its capacity to scavenge reactive oxygen and nitrogen species and protect cells/organism from oxidative damage and thus could be an effective against oxidative stress. In addition, the *Cissus vitiginea* leaf found to contain a noticeable amount of total phenols which plays a major role in controlling antioxidants. Thus, it can be concluded that *Cissus vitiginea* leaf can be used as an accessible source of natural antioxidants with consequent health benefits.

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