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EVALUATION OF THE FREE RADICAL SCAVENGING ACTIVITY OF FOUR VARIOUS SOLVENT EXTRACTS FROM *CLEOME VISCOSA* WHOLE PLANT

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Abstract
Free radicals produced and their increased levels by a variety of metabolic actions in the body. This increased levels of free radicals lead to several diseases. As a result, it is essential for defusing the overload of free radicals. The purpose of the present study is to establish the antioxidant activities of methanolic extract (MeCV), aqueous extract (AqCV), ethyl acetate extract (EaCV) and n-hexane extract (NhCV) of *Cleome viscosa* whole plant on hydroxyl radical, total antioxidant activity, ferrous chelating, nitric oxide and singlet oxygen scavenging, and reducing power assays were estimated. Results of our presented study revealed that, MeCV shown better antioxidant activity among the extracts with IC_{50} values 60.27±1.07µg/mL (hydroxyl radical scavenging) 43.85±1.56µg/mL (Total antioxidant activity), 94.47±1.20µg/mL (Ferrous ion chelating), 39.36±0.75µg/mL (nitric oxide scavenging) and 81.64±1.53µg/mL (singlet oxygen scavenging). The reducing power was improved with increasing concentration of plant extracts. The results concluded that, MeCV might be potent extract in *C. viscosa*, and it had high antioxidants which may be associated with its ethnomedicinal use.

Keywords: *Cleome viscosa*, Antioxidant activity, Nitric oxide scavenging, solvent extracts, singlet oxygen scavenging.

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INTRODUCTION

Chemical compounds exhibit reductive assets have been lengthy used in food production to defend foods against oxidation; still, the current interest branch from their capability to defend oxidative stress in the human organism. Oxidative stress (OS) is a difference among antioxidant defense system and the fabrication of ROS. The combined term "ROS" consist of both free radicals [molecules have an abnormal electron, like superoxide anion radicals (O_2•−) and hydroxyl radical (•OH)] and the group that are not free radicals, like singlet oxygen ($^1O_2$) and ozone (O_3) [1]. There are many methods to estimate the *in vitro* antioxidant ability of plant extracts, combination of compounds, biological solutions and tissues which engage particular system of determination of antioxidant action, for example: chemical system based on ROS scavenging, such as the hydroxyl radical (OH·) and nitric oxide (NO·) radical [2]. Additional assays to conclude the total antioxidant activity consist of a method such as TAC assay (phosphomolybdenum method) [3]. Different reaction mechanisms are regularly implicated to evaluate the antioxidant ability of natural antioxidants and presently no single broad-spectrum method which can confirm absolute, accurate and quantitative estimation of antioxidant significance and anti-radical capability, consequently, more than one method is recommended to estimate the antioxidant activities [4]. Antioxidants are important material that plays an important role in postponement, intercepting, and avoid oxidative effect catalyzed by free radicals, as a result, a condition is established in the protection of humans [5]. Owing to this special capability there is a better use of antioxidants for the stability of ROS. At present days, the majority of the antioxidants are artificially synthesized. A number of synthetic antioxidants like Gallic acid, BHA (Butylatedhydroxyl anisole), BHT (Butylated hydroxyl toluene), and TBHQ (Tertiary butylated hydroxyl quinone) are commercially available. Such types of synthetic antioxidants are well-known to contain probable side effects and have some quantity of carcinogenicity while in use *in vivo* [6,7]. Therefore their application or use is restricted to few organisms. Plant materials that contain antioxidant substance could protect and prevent free radicals produced, in this manner defending the organism from different diseases. For that reason, a special interest to search medicinal plants that contains more amounts of natural antioxidants has significantly increased. Plant derivative natural compounds such as flavonoids, alkaloids, terpenes, etc, have acknowledged significant consideration in last few years due to their pharmacological benefits including Antioxidant, Antimicrobial and Anti-inflammatory activities [8-10]. *Cleome viscosa L.* plant belongs to family "Capparidaceae". It is commonly known as Asian spider flower or tick weed is an annual herb, grows to a meter height. The roots and leaves contain the source of flavonoids,

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alkaloids, phenols, aromatic amines and alkynes, amino acids. The Seeds Contain 26% oil, palmitic acid stearic acid, oleic acid and linoleic acid [11]. In Indian- Chinese traditional medicine of ayurvedic system this plant is an important medicine for malaria, fever, piles, snake bites and hypertension [12]. *Cleome viscosa* plant various parts possesses anti-inflammatory, antimicrobial [13], antidiarrheal [14], antimalarial [15] activities. In the present study, we determined the *in vitro* antioxidant activity of MeCV, AqCV, EaCV and NhCV from *C. viscosa* whole plant by using hydroxyl radical scavenging, total antioxidant capacity, ferrous ion chelating, nitric oxide scavenging, singlet oxygen scavenging and reducing power methods based on different mechanisms of determination of the antioxidant ability in comparison with standard reference compounds.

MATERIALS AND METHODS

Chemicals

All chemicals used including the solvents were of analytical grade. Ascorbic acid, EDTA, and lipoic acid were purchased from Merck (Tirupati, India). All other chemicals and reagents used were of the highest commercially available purity. The solvents methanol, ethyl acetate and n-hexane were purchased from Mercury commercial sources (Tirupati, India).

Plant material collection and identification

The whole plant of *Cleome viscosa*, were collected from Dravidian University surroundings, Kuppam, Andhra Pradesh, India. Taxonomic identification was made by Prof N. Yasodamma, Department of Botany, S.V. University, Tirupati, India. The plant material were shade dried and powdered by a mechanical grinder.

Preparation of extracts

The powder materials were successively extracted by using Soxhlet apparatus for 6 hrs with methanol, aqueous, ethyl acetate and n- hexane solvents (1:5 ratio W/V) was extracted by using were concentrated to dehydration under the vacuum 70-80^0 c. The extracts were concentrated by using a rotary evaporator and subjected to freeze-drying in vacuum at 35º-40º c and dry powder was obtained.

Evaluation of *in vitro* antioxidant activity

Hydroxyl radical scavenging activity

0.1 ml of different concentrations (20-100 µg/mL) of four various extracts were incubated with 0.3 mL of deoxyribose (30 mM), 0.3 mL of hydrogen peroxide (20 mM), 0.3 mL ferric chloride (of 1 mM), 0.3 ml of EDTA (1 mM), 0.3 ml of and ascorbic acid (1 mM) in 1.4 ml of potassium phosphate buffer (50 mM, pH 7.4) at 37°C for 60 min. The reaction was ended by adding 0.5 ml of TCA (5% solution) followed by addition of 0.5 ml of TBA (0.2% solution),

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and again boiling in a water bath for 15 min. The absorbance was deliberate at 535 nm. Ascorbic acid was used as the reference control [16]. The percentage of hydroxyl radical inhibition was determined by using the following formula;

% hydroxyl radical scavenging = $[(A_0 - A_1) / A_0] \times 100$

Where,

A_0 = absorbance of the control,

A_1 = absorbance in the sample and standard.

Total Antioxidant Capacity (TAC)

The total antioxidant capacity (TAC) of four various extracts of *C. viscosa* was spectrophotometrically resolute by the phosphomolybdenum method by using the method described by Prieto *et al.* 1999 [17]. Briefly, 0.3 mL (20-100 µg/mL) of a 1 mg/mL (solution in methanol) extracts was mixed with 2.7 mL of phosphomolybdenum reagent (28 mM sodium phosphate and 4 mM ammonium molybdate in 0.6 M sulphuric acid) in ceiling test tubes. Incubation was then carried at 95° C for 90 min in a water bath. After cooling to room temperature, the absorbance of the reaction mixture was measured at 695 nm by using a UV-visible spectrophotometer against a 0.3 mL of a methanol solution of blank (without plant extract). TAC results were calculated by using the following formula; Ascorbic acid was used as a standard for comparison.

% TAC = $[(A_0 - A_1) / A_0] \times 100$

Where,

A_0 = absorbance of the control,

A_1 = absorbance in the sample and standard.

Ferrous Ion Chelating Assay

The ferrous ions (Fe^{2+}) chelation was evaluated by the method modified by Dinis *et al* 1994 [18]. Briefly, different concentrations 20-100 µg/mL of each extract (1 mg/mL) was taken and makeup to 3 mL of methanol. 20 µL of 2 mM FeCl2 was added with 740 µL of methanol. The reaction was started by the adding together of 40 µL of 5 mM ferrozine into the mixture, which was then deficient for 10 min at room temperature and then the absorbance was determined at 562 nm. In this assay, the blank sample was prepared by using 20 µL milliQ purified water instead of ferrozine. EDTA was used as positive control. The percentage of inhibition of Fe^{2+}–ferrozine complex formation was calculated formula was given below:

% ferrous ion chelation scavenging = $[(A_0 - A_1) / A_0] \times 100$

Where,

A_0 = absorbance of the control,

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A_1 = absorbance in the sample and standard.

Nitric oxide radical scavenging activity

Nitric oxide produced on sodium nitroprusside in aqueous solution at pH interacts with oxygen to generate nitrite ions, which were deliberated by the method of Garrat 1964 [19]. 3 mL of reaction mixture containing various concentrations (20-100 µg/mL) of 0.5 mL of four various extracts, 2 mL of 10 mM sodium nitroprusside and 0.5 mL of 1M phosphate buffer saline were incubated at 25^0C for 150 mins. After incubation, 0.5 mL of the reaction mixture containing nitrite was pipette out and mixed with 0.33 % of 1 mL sulphanilic acid reagent and allowed to stand for 5 min for implementation of diazotization. Then 1 % of 1 mL naphthylethylene diamine dihydrochloride was added, mixed well and allowed to stand 30 mins for reaction. The absorbance was deliberate at 546 nm. The percentage inhibition of nitric oxide radicals scavenging was obtained by using the given formula;

% of scavenging = $[(A_0 - A_1) / A_0] \times 100$

Where,

A_0 = absorbance of the control,

A_1 = absorbance in the sample and standard.

Singlet oxygen scavenging assay

Singlet oxygen ($^1O_2$) production, and at the similar time, its scavenging ability by the test samples and the reference compound lipoic acid can be observed by *N*, *N*-dimethyl-4-nitrosoaniline (RNO) peroxide, using a previously described method [20]. Singlet oxygen ($^1O_2$) was generated by a reaction between NaOCl and H_2O_2 and the bleaching of RNO was read at 440 nm. The reaction mixture contained 45 mM of phosphate buffer (pH 7.1), 50 mM of NaOCl, 50 mM of H_2O_2, 50 mM of histidine, 10 µM of RNO and various concentrations (20–100 µg/mL) of four various extracts in a final volume of 2 mL. It was incubated at 30° C for 40 min and the scavenging activity of the sample was compared with that of the lipoic acid reference compound. Singlet oxygen scavenging was calculated by the following formula:

% of scavenging = $[(A_0 - A_1) / A_0] \times 100$

Where,

A_0 = absorbance of the control,

A_1 = absorbance in the sample and standard.

Reducing power assay

The reaction mixture contained 2.5 mL of various concentrations (20-100 µg/mL) of four various extracts of the test sample, 2.5 mL of 1% potassium ferric cyanide and 2.5 mL of 0.2 M sodium phosphate buffer, [21]. The control consisted all the reagents in the absence of test

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sample. The combination was incubated at 50^0 C for 20 min and was ended by the addition of 2.5 mL trichloroacetic acid (10% w/v), pursue by centrifugation for 10 minutes at 3000 rpm. 5 mL of the supernatant was separated and mixed with 5.0 mL distilled water and add 0.5 mL of 0.1 % ferric chloride, and absorbance was measured at 700 nm against a blank (distilled water and phosphate buffer contained). Ascorbic acid was used as standard reference drug.

Statistical analysis

All the assays were carried out in triplicate. Experimental results are expressed as mean± standard error. The results were analyzed using one-way analysis of variance and the group means were compared using P<0.05 followed by Tukey's post hoc test and *P<0.01 and **P<0.001 was Performed One Way ANOVA followed by Dunnett test using SPSS version 16. The percentage of scavenging activity graphs were plotted by MS-excel.

RESULTS AND DISCUSSION

Table 1: In vitro Antioxidant activity of different extracts obtained from *Cleome viscosa* whole plant

	IC_{50} Values (µg/ml)						
Free radical scavenging assays	**Plant Extracts and Reference Compounds**						
	EDTA	**Ascorbic acid**	**Lipoic acid**	**MeCV**	**AqCV**	**EaCV**	**NhCV**
Hydroxyl radical	-	53.14±1.25^a	-	60.27±1.07^b	65.59±1.25^b	74.13±2.10^c	80.70±1.52^c
Total antioxidant	-	46.00±0.74^a	-	43.85±1.56^a	45.71±0.65^a	54.15±1.45^b	61.02±0.65^c
Ferrous chelating	72.26±0.89^a	-	-	94.47±1.20^b	96.93±1.55^b	109.75±1.44^c	114.09±1.65^c
Nitric oxide	-	24.32±0.14^a	-	39.36±0.75^b	45.23±0.67^c	68.72±01.03^d	72.54±01.20^d
Singlet oxygen	-	-	44.47±1.00^a	81.64±1.53^b	85.23±0.82^b	117.55±2.87^c	121.16±2.64^c

Values in the table are represented as mean ± SEM for triplicates

Means ±SEM not sharing a common superscript in the row differ significantly at P<0.05 (Performed One Way ANOVA followed by Tukey's post hoc).

Hydroxyl radical scavenging

Hydroxyl radical is an enormous and immediate free radical produced in genetic organization and has been occupied as vastly destructive species in free radical pathology, competent of destructive about each molecule; proteins, DNA, lipids and unsaturated fatty acids in approximately every biological membrane originate in living cells [22]. Hydroxyl radical scavenging activity was enumerated by determines the inhibition of the removal of 2-

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deoxyribose by the free radicals fabricated by the Fenton reaction. In the year of 2000, Cheng and Breen are a statement the capacity of flavonoids to suppress the Fenton reaction [23]. In our results represents, the hydroxyl radical scavenging activity of *C. viscosa* whole plant extracts and standard ascorbic acid can be ranked as ascorbic acid > MeCV > AqCV > EaCV and NhCV (Table 1). All extracts showed antioxidant activity in concentration dependent behavior at concentrations 20-100 µg/mL (Figure 1). In the present study, the IC_{50} values of hydroxyl radical scavenging activity for the MeCV and AqCV was 60.27±1.07 and 65.59±1.25 µg/mL while for EaCV and NhCV was 74.13±2.10 and 80.70±1.52 µg/mL (Table 1). The noticeably strong ($P<0.05$) antioxidant response of MeCV in comparison with ascorbic acid may be helpful in differentiating the major sources of natural antioxidant effect. Several studies are displayed that major group (high polarities of compounds) of phytochemicals inhibited production of free radicals. Moreover, the occurrence of phenols, flavonoids and catechol group in terpenoids as well helped to entrap and alleviate the free radicals [24].

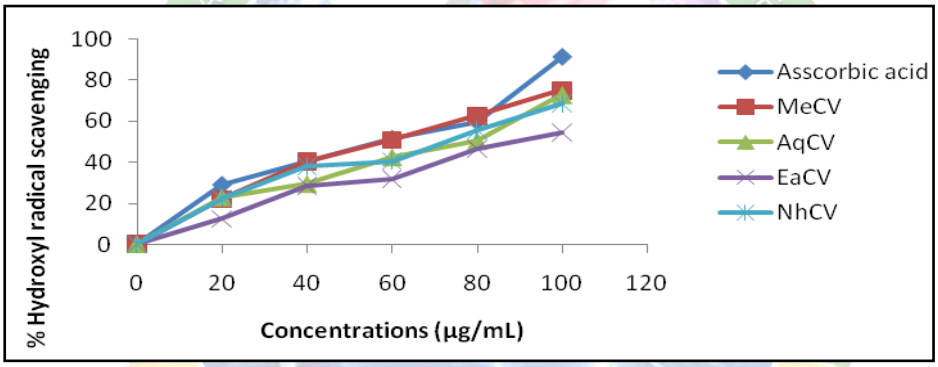

Fig. 1: Hydroxyl radical scavenging activity of four various extracts of *Cleome viscosa* whole plant.

Total antioxidant activity

The phosphomolybdate method is significant, while the total antioxidant capacity (TAC) is compared to ascorbic acid. It is a calorimetric quantitative technique which dealings the decrease of Molybdenum blue to form of molybdenum (V) phosphate by the model and following the development of a bluish green color Phosphate-Mo (V) complex [25]. It facilitates to scrutinize the decrease rate of antioxidants and molybdenum ligand. In the present study, capacity of antioxidant activity in four various extracts of *C. viscosa* was originate to decrease in this order: ascorbic acid > MeCV > AqCV > EaCV > NhCV (Table 1; Figure 2). All extracts showed antioxidant activity in concentration dependent manner at concentrations 20-100 µg/mL. The IC_{50} values of antioxidant capacity for the MeCV (43.85±1.56 µg/mL) were most potent ($P< 0.05$) than EaCV (54.15±1.45 µg/ml) and NhCV (61.02±0.65 µg/mL). The strong antioxidant activity of MeCV statistically similar to standard ascorbic acid indicates controlling antioxidants in this extract and these might be attributed to

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the occurrence of phytochemical compounds. According to present results, a highly optimistic association among phytochemicals and antioxidant action shown to be there the trend in several plant species [26].

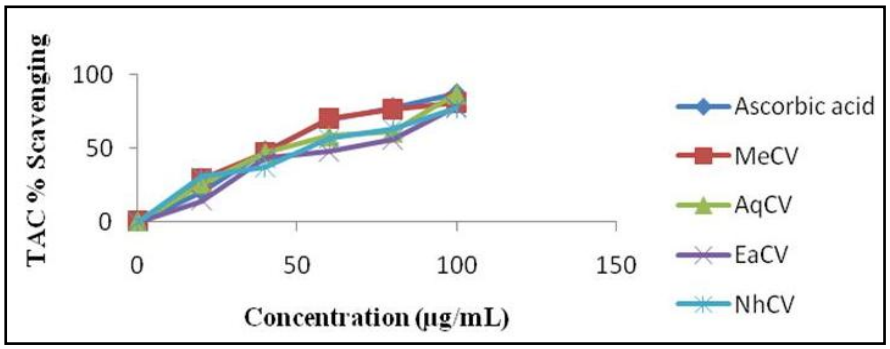

Fig. 2: Total antioxidant activity (TAC) of four various extracts of *Cleome viscosa* whole plant.

Ferrous ion chelating ability assay

The ferrous ions chelating activity of MeCV, AqCV, EaCV and NhCV of *C. viscosa* whole plant and standard EDTA were investigated (Figure 3). Ferrous ions chelation may give important antioxidative property by interruption of metal-catalyzed oxidation. Ferrous ions are the mainly commanding pro-oxidants among the diverse species of metal ions. Reduced ferrous (Fe^{2+}) ions can afford a defense against oxidative damage by inhibiting fabrication of ROS and lipid peroxidation [27]. In our results, MeCV, AqCV, EaCV and NhCV showed 52.98, 51.85, 45.09 and 44 % ferrous ion chelating capability at 100 µg/mL of higher concentration and the EDTA showed 58.76 % at the same concentration. The IC_{50} value of the MeCV was also good quality when significantly ($p<0.05$) comparison with the reference standard EDTA IC_{50}value of (Table 1). The IC_{50} values MeCV, AqCV, EaCV and NhCV of *C. viscosa* and reference compound EDTA were found to be 94.47±1.20, 96.93±1.55, 109.75±1.44 and 114.09±1.65 µg/mL and 72.26±0.89 µg/mL, respectively. Similar kind of results [28] reported that p-coumaric acid and Coffee brews [29] exhibited a high scavenging effectiveness toward the ability of ferrous ion chelation.

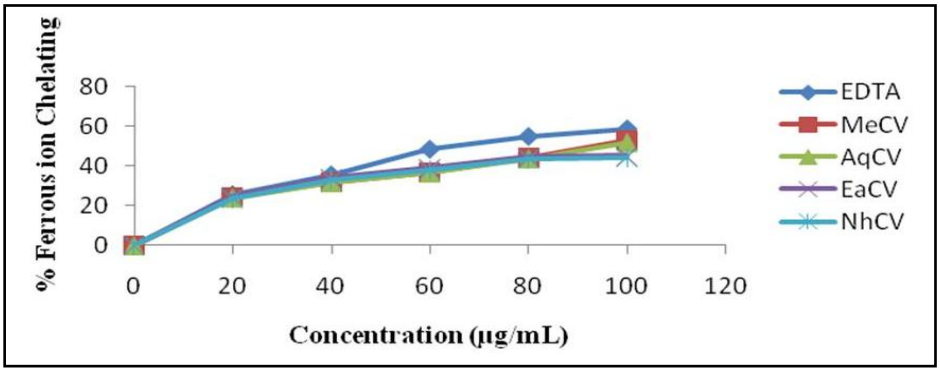

Fig. 3: Ferrous ion chelating activity of four various extracts of *Cleome viscosa* whole plant.

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Nitric oxide radical scavenging

Nitric oxide scavenging activity was executed in four different extracts (20-100 µg/mL) of *C. viscosa* by using Ascorbic acid as a standard antioxidant (20-100 µg/mL). Higher levels of hydroxyl radicals are poisonous to tissue and that leads to the vascular collapse, an assortment of inflammations, and ulcerative colitis. The nitric oxide toxicity increases when it responds with superoxide radicals and form extremely reactive peroxy nitrate anion [30]. Therefore, in our results represents the MeCV, AqCV, EaCV and NhCV of *C. viscosa* were found to be greatly proficient in the percentage of nitric oxide scavenging (Figure 4) among these MeCV displayed the highest activity. The IC_{50} values for MeCV, AqCV, EaCV and NhCV were 39.36±0.75, 45.23±0.67, 68.72±01.03 and 72.54±01.20 µg/mL respectively. Ascorbic acid was used as a reference compound which has an IC_{50} value of 24.32±0.14 µg/mL. At 100 µg/mL, the percentage inhibition of MeCV, AqCV, EaCV and NhCV were 73.37, 65.14, 62.21 and 50.63% respectively while for the standard ascorbic acid the percentage inhibition was 76.53%. In the previous study, *Centella asiatica* [31] and *Newbouldia laevis* [32] plants have shown similarly significant effect.

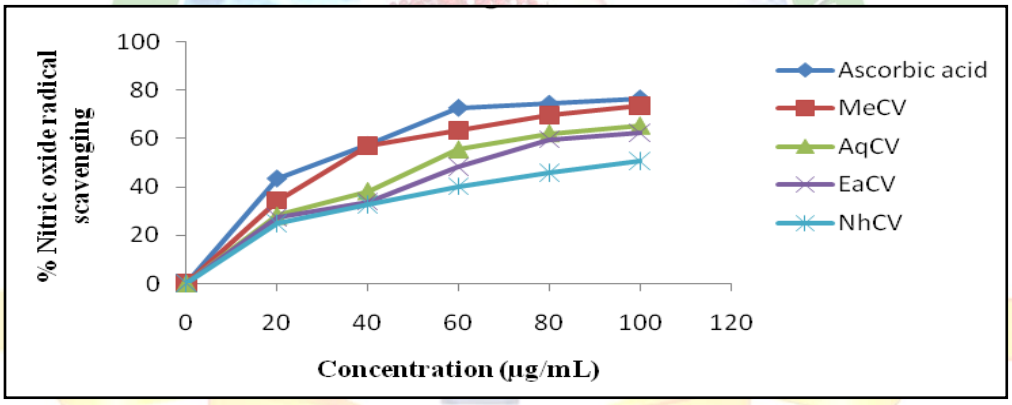

Fig. 4: Nitric oxide free radical scavenging activity of four various extracts of *Cleome viscosa* whole plant.

Singlet oxygen scavenging assay

Singlet oxygen is produced in the skin membrane during ultraviolet radiation. It is an elevated energy structure of oxygen and is acknowledged as being of the ROS. Singlet oxygen stimulates hyper oxidation and oxygen cytotoxicity and reduces antioxidative action [33]. The current study indicates that the MeCV, AqCV, EaCV and NhCV extracts also showed reasonable dose-dependent scavenging results of singlet oxygen species. MeCV extract was a valuable scavenger of singlet oxygen (figure 5) and this action was analogous to that of standard lipoic acid. The IC_{50} values (Table 1) of the MeCV, AqCV, EaCV and NhCV sample was 81.64±1.53, 85.23±0.82, 117.55±2.87 and 121.16±2.64 µg/mL whereas that of the standard lipoic acid compound was 44.47±1.00 µg/mL. The IC_{50} value of the MeCV was

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higher than the remaining three extracts. At 100 µg/mL, the percentage scavenging capacity of the MeCV was 60 % whereas that of lipoic acid was 77.09 %. Interestingly, relatively an only some studies on the antioxidant assets of the two plant resources, viz., *Newbouldia laevis* and *Amazonian plants* [34] have been done earlier.

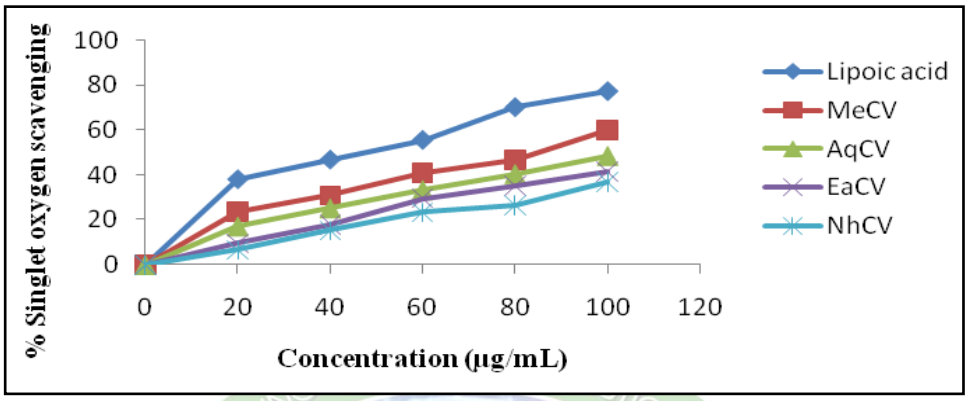

Fig. 5: Singlet oxygen scavenging activity of four various extracts of *Cleome viscosa* whole plant.

Reducing power assay

MeCV, AqCV, EaCV and NhCV extracts of *C. viscosa* displayed significant reducing power (Table.2). Reducing power is a single method for the act of antioxidants and might serve up as an important marker of probable antioxidant action for antioxidants [35]. Increased absorbance at 700 nm indicated an increase in reducing power ability. Table 2 shows dose-response manner for the reducing ability of the four extract. The absorbance of gallic acid, MeCV, AqCV, EaCV and NhCV at highest concentration (100 µg/mL) was found to be 0.76±0.014, 0.51±0.005, 0.40±0.004, 0.36±0.004, and 0.28±0.009 respectively. The highest reducing power of *C.viscosa* four extracts were shown in MeCV, followed by AqCV, EaCV and NhCV, respectively. The MeCV showed good reducing power ability that was comparable with that of gallic acid. The antioxidant activity was confirmed by this medicinal plant as occurring natural antioxidants. Some studies have shown that the antioxidant result is associated with the progress of reductones [36].

Table 2: Reducing power of different extracts obtained from *Cleome viscosa* whole plant.

	Different concentration(µg/ml)				
Plant Extracts/ Standard	**20**	**40**	**60**	**80**	**100**
Gallic acid	0.31±0.005	0.41±0.006	0.54±0.012	0.61±0.010	0.76±0.014
MeCV	0.20±0.005*	0.24±0.014**	0.32±0.024**	0.43±0.022**	0.51±0.005**
AqCV	0.17±0.011**	0.21±0.009**	0.31±0.017**	0.34±0.004**	0.40±0.004**
EaCV	0.15±0.023**	0.18±0.003**	0.24±0.176**	0.30±0.014**	0.36±0.004**
NhCV	0.13±0.013**	0.17±0.011**	0.18±0.081**	0.20±0.007**	0.28±0.009**

Values in the table are represented as mean ± SE for triplicates

Means in the column differ significantly compared to standard (Gallic Acid) at *P<0.01 and **P<0.001 and (Performed One Way ANOVA followed by Dunnett test)

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CONCLUSION

Depending on the results, in the current study, it can conclude that MeCV exhibits prominent antioxidant and free radical scavenging activities. It also chelates ferrous ion and has reducing power. These *in vitro* assess indicate that, MeCV is an important source of natural antioxidant, which may be supportive in impeding the development of different oxidative stresses. However, the mechanism responsible for the anti-radical activities is presently undecided. Therefore, more investigation is required to isolate and categorize the antioxidant complex present in the MeCV.

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