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ANTIOXIDATIVE PROPERTIES (TPC, DPPH, FRAP, METAL CHELATING ABILITY, REDUCING POWER AND TAC) WITHIN SOME *CLEOME* SPECIES

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Abstract – Applications of antioxidants are increasing due to their multiple roles in removing harmful effects of oxidative stress. For the present study TPC, DPPH, FRAP, Metal Chelating Ability, Reducing Power and TAC spectrophotometric assays were used for the assessment of antioxidative properties of methanolic extracts of leaves of 5 different *Cleome* species (*Cleome viscosa* L., *C. simplicifolia* (Camb.) Hook f. & Thoms, *C. gynandra* L., *C. chelidonii* L.f. and *C. speciosa* Raf.). The purpose of this study was also to evaluate the comparability of antioxidant capacity measurements in *Cleome* species by the above assay. This study may provide some supplementary food option by some *Cleome* species.

Keywords: Antioxidant property, Chelating activity, *Cleome*, Reducing power

Introduction

An antioxidant can be defined as ‘‘any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate’’ (Halliwell & Gutteridge, 1999). The concept of antioxidant capacity was first originated from chemistry and was later established to biology, medicine, epidemiology and nutrition. Antioxidants are considered important nutraceuticals on account of their many health benefits and are widely used in the food industry as inhibitors of lipid peroxidation (Scherer & Godoy, 2009). Synthetic antioxidants, accumulate in the body causing liver damage and carcinogenesis. Such problems are not seen when natural antioxidants extracted from herbs and spices are used. These extracts are safe, potentially nutritional and have therapeutic effects. Plant material such as vegetables, fruits, seeds, woods, barks, roots, leaf spices, and herbs have been examined as potential source of antioxidants (Velioglu et al., 1998; Rubilar et al., 2006).

Since most antioxidant compounds are introduced to the organism through diet, it is desirable to establish methods that can directly measure the total antioxidant capacity of food plant extracts (Ou et al., 2002). In the present study, antioxidant activities were evaluated in *Cleome* species through DPPH and FRAP assays, TAC, Ferrous ion chelating ability and Reducing Power assay.

Materials and methods

The locally collected seeds of *Cleome* spp. (*Cleome viscosa* L., *C. simplicifolia* (Camb.) Hook F. & Thoms, *C. gynandra* L., *C. chelidonii* L.F. and *C. speciosa* Raf.) were sown in pots and plots. The seedlings were raised in fertile soil in earthenware pots and allowed to grow and establish under normal conditions with proper irrigation. The leaves of all these species were collected freshly and used for further analysis.

The polyphenols were estimated following the method of Folin & Denis (1915) from the acetone extract.

The antioxidant activities of plant extracts and the standard were assessed on the basis of the free radical scavenging

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effect of the stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH)-free radical activity (Braca et al., 2002). The diluted working solutions of the test extracts were prepared in methanol. Ascorbic acid (10mg/ml) was used as standard. 0.002% DPPH was prepared in methanol and 3 ml of this solution was mixed with 1 ml of sample solution and standard solution separately. These solution mixtures were kept in dark for 30 min and optical density was measured at 517 nm using Spectrophotometer. Methanol (1 ml) with DPPH solution (0.002%, 3 ml) was used as blank. The optical density was recorded and % inhibition was calculated using the formula given below:

$$\text{Percent inhibition of DPPH activity} = \frac{absorbance(cont)-absorbance(sam)}{absorbance(cont)} \times 100$$

Antioxidant activity assay was also done following the Ferric-reducing antioxidant power (FRAP) method described by Benzie & Strain (1996). The results are expressed as ascorbic acid equivalent antioxidant capacity.

The ferrous ion-chelating ability was determined according to the method of Decker & Welch (1990) with minor modifications. 1.6ml of each extract stock solution (0.2, 0.4, 0.6, 0.8 and 1 mg/ml) were mixed with 2.16 ml of distilled water and 80 µl of 2 mM $FeCl_2$ in a test tube. The reaction was initiated by the addition of 160 µl of 5 mM ferrozine. The solutions were well mixed and allowed to stand for 10 min at room temperature. After incubation, the absorbance was measured at 562 nm spectrophotometrically. Distilled water (1.6 ml) instead of sample solution was used as a control. Distilled water (160 µl) instead of ferrozine solution was used as a blank, which was used for error correction because of unequal colour of the sample solutions. L-ascorbic acid was used as reference standard. All measurements were performed in triplicate. The ferrous ion-chelating ability was calculated as follows:

$$\text{\% scavenging activity (Ferrous ion chelating ability)} = \frac{(Ac - AE/As)}{Ac} \times 100$$

Where, Ac is the Absorbance of Control reaction; AE is the Absorbance of plant extract; As is the Absorbance of standard.

The reducing power was determined according to the method (Reducing Power Assay) of Oyaizu (1986). An aliquot of 0.5 ml plant extract (0.2, 0.4, 0.6, 0.8, 1 mg/ml) was mixed with 1 ml phosphate buffer (0.2M, pH 6.6) and 1 ml 1% K3Fe(CN)6, shaken well and incubated at 50^0C for 20 min. After incubation, 1 ml TCA(10%) was added to stop the reaction. It was centrifuged at 3000rpm for 10 min. 1.5 ml supernatant, 1.5 ml D.W. and 0.1 ml FeCl3 (0.1%) were mixed and incubated for 10 min and absorbance was read at 700nm on Spectrophotometer .The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The extract concentration providing 0.5 of absorbance (EC50) was calculated from the graph of absorbance at 700 nm against extract concentration. Ascorbic acid was used as standard. Higher absorbance indicates higher reducing power.

The total antioxidant capacity of methanol extracts of leaves of *Cleome* species was evaluated by the phosphomolybdenum method of Prieto et al. (1999). An aliquot of 0.5 ml of sample solution (0.2, 0.4, 0.6, 0.8, 1 mg/ml) was combined with 5 ml reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a boiling water bath at 95^0C for 90 min. After the samples had cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against blank on Spectronic 20 visible spectrophotometer. A typical blank solution contained 5 ml of reagent solution and the appropriate volume of the same solvent (methanol) used for the sample and it was incubated under same conditions. For samples, antioxidant capacity is expressed as equivalents of ascorbic acid.

RESULTS AND DISCUSSION

Total polyphenol contents

Polyphenols represent the aromatic compounds formed during secondary metabolism in plants. These phenolic compounds, in some instance, affect the fundamental plant processes such as photosynthesis, chlorophyll synthesis, water relations, protein synthesis, respiration, membrane permeability, etc. The phenols and their oxidative products are also known for inhibitory action on various enzyme systems e.g. IAA-oxidase (Shekhawat et al., 1980). As polyphenols are known to accumulate during the fungal attack, their role in disease resistance has also been well demonstrated. The importance of polyphenols can be very well recognized at the time of stress.

In pharmacological studies, polyphenols are considered as secondary products of plant metabolism without specific physiological function (Adzet, 2002). Polyphenols show natural antioxidant properties, therefore impart a tonifying action (Thompson & Williams, 1976). Some flavonoids act as powerful electron scavengers of free radicals (Rice-Evans

et al., 1997) and electron doners to the H_2O_2 scavenging peroxidases of plant cells. The total polyphenol content of young, mature and senescent leaves of five *Cleome* species is recorded in Fig.1. It is evident that the senescent leaves of all species of *Cleome* contain the highest amount of total polyphenols as compared to that in mature and young leaves. In all *Cleome* species total polyphenol content of the developing leaves changes with similar pattern i.e. YL<ML<SL. The leaves of *Cleome viscosa* have recorded the maximum amount of polyphenols while those of *Cleome speciosa* posses the minimum amount of polyphenols.

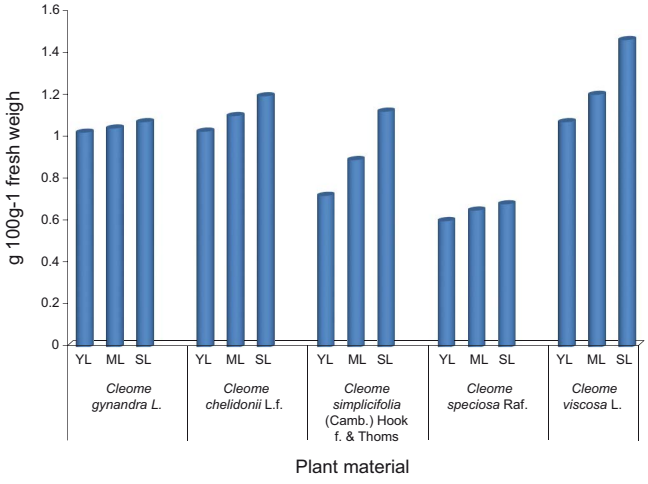

Figure 1. Total polyphenol contents of the leaves of *Cleome* species.

DPPH radical scavenging activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH), is a kind of stable organic radical. The capacity of biological reagents to scavenge DPPH radical, can be expressed as its magnitude of antioxidation ability. The DPPH oxidative assay (Peng et al., 2000) is used worldwide in the quantification of radical-scavenging capacity. The antioxidant activities of plant extracts and the standard were assessed on the basis of the free radical scavenging effect of the stable DPPH free radical activity (Braca et al., 2002). The results are expressed as the IC50 value (the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%).

The changes in the free radical scavenging ability of methanolic extracts of leaves of five *Cleome* species on the basis of percent inhibition is presented in Fig 2. It is evident from the figure, that the methanolic extract of *Cleome chelidonii* has the highest and that of *Cleome viscosa* has the lowest free radical scavenging potential among the *Cleome* species studied.

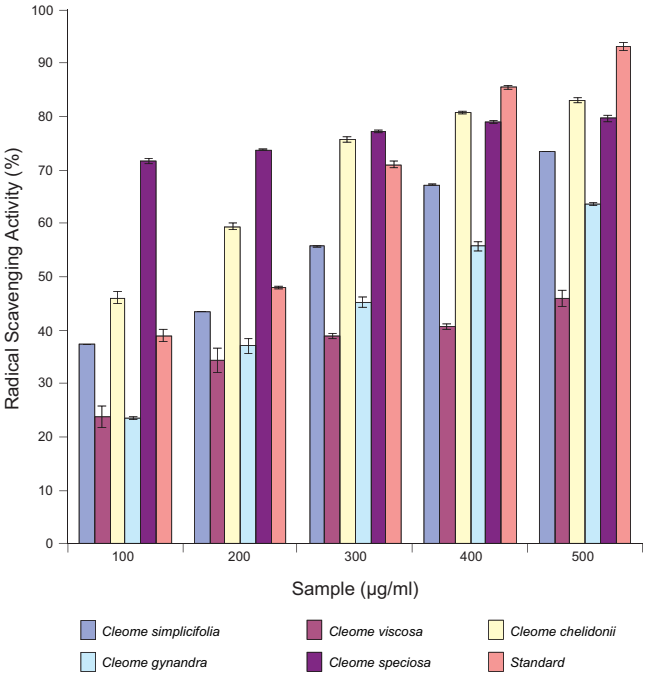

Figure 2. Radical scavenging activity by DPPH method.

In the present study the leaves of *Cleome chelidonii* have shown a high antioxidant activity which can be attributed to its high TPC (total phenolic content), however, *Cleome viscosa* has lower radical scavenging activity which might be exactly opposite to its TPC value. According to Bondet et al. (1997) the reaction of DPPH with certain phenols is reversible, resulting in low readings for antioxidant activity. The order of antioxidant activity in Cleome species can be given as *C. chelidonii* > *C.speciosa* > *C. simplicifolia* > *C. gynandra* > *C. viscosa*..

The results of the DPPH free radical scavenging assay suggest that leaves of all *Cleome* species have potent antioxidant property of scavenging free radicals. These species could be used as a potent source for the cancer chemo protective therapy.

Ferric-reducing antioxidant power (FRAP)

The ferric reducing antioxidant plasma activity of *Cleome* leaves is presented in Fig 3. It is clear from the results that the FRAP activity of leaves of *Cleome speciosa* is the highest among all the *Cleome* species studied.

Wootton-Beard et al. (2011) have determined the FRAP values for 23 commercially available vegetable juices. James White organic vegetable juice had this value as 2601±22 µmol/L, it was 2573±49 µmol/L for V8 100% vegetable juice (original) and 2468±93 µmol/L for Eden organic vegetable

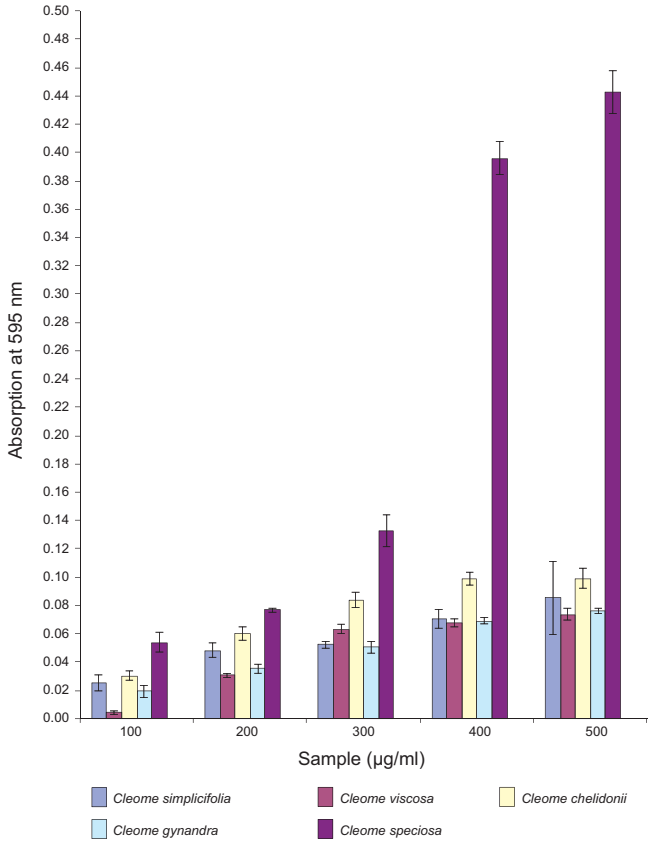

Figure 3. Antioxidant activity in some *Cleome* species by FRAP method.

cocktail. Katalinic et al. (2006) screened seventy medicinal plant extracts for antioxidant capacity and total phenols. The FRAP values ranged from 0.06 to 25 mM/L. According to their antioxidant capacity, the medicinal plant extracts were divided in to five groups: (a) very low FRAP (<1 mM/L) n = 9; (b) low FRAP (1–5 mM/L), n = 37; (c) good FRAP (5–10 mM/L), n = 15; (d) high FRAP (10–20 mM/L), n = 8; and (e) very high FRAP (>20 mM/L), n = 1. The best results were obtained for *Melissae folium* which showed a very high FRAP (>20 mM/L) values. The antioxidant activities of various cultivars of onion were carried out by Gokce et al. (2010). Red cultivar contained 9.3 mmol TE/g dw FRAP and yellow onions had this value as 9.8 mmol TE/g dw which were much higher than that for white variety. Liu et al. (2009) determined the polyphenolic content and antioxidant activity of leaves of *Camellia sinensis.* The antioxidant activities of crude extract and its fractions decreased in the order, ethanolic extract 6.7 > n butanolic fraction 4.7 > chloroform fraction 4.2 ± 0.2 > water fraction 1.5 ± 0.1 m mol FeSO4/g DW. Total equivalent antioxidant capacities (TEAC) and phenolic contents of 32 species extracts from 21 botanical families grown in Poland were investigated by Wojdylo et al. (2007). Their FRAP values ranged from 13.8 to 21.33 µM trolox/100 g DW.

Surveswaran et al. (2007) evaluated one hundred thirty two medicinal plants for the antioxidant activities of leaves. Following are some of these plants with their antioxidant activity. *Aloe littoralis* Baker 8.68, *Murraya exotica* L. 1.80, *Vitex negundo* 1.44 and *Viola serpens* Wall. ex Ging. 0.91 µmol/g DW. Konczak et al. (2010) have determined antioxidant capacity and phenolic compounds in commercially grown native Australian herbs and spices. Anise myrtle (*Syzygium anisatum*) exhibited the highest total reducing capacity (Ferric Reducing Antioxidant Power assay), followed by *Tasmannia* pepper leaf and lemon myrtle (*Backhausia citriodora*). Andlauer & Héritier (2011) focused their studies on antioxidant capacity of tea samples. Their samples with FRAP values were as follows: Green tea I 1.146 ± 0.016, Green tea II 1.053 ± 0.024, Green tea III 1.098 ± 0.023, Yellow tea 0.709 ± 0.010, White tea 0.484 ± 0.002, Oolong Chinese 0.319 ± 0.002 and Oolong Vietnamese 0.198 ± 0.002 mg TE/mL.

The highest absorbance of FRAP was observed in *Cleome speciosa* at 1000 µg/ml and the lowest was that in *Cleome viscosa* at 200µg/ml i.e 0.443±0.0069 and 0.0043±0.0012 respectively. When all *Cleome* species are compared for each concentration the following pattern of absorbance can be observed *C. speciosa* > *C. chelidonii* > *C. simplicifolia* > *C. gynandra* > *C. viscosa.* In all *Cleome* species 800 µg/ml and 1000 µg/ml concentrations are effective to react with ferric tripyridyltriazine (Fe^{III}- TPTZ) complex and produce a colored ferrous tripyridyltriazine (Fe^{II}-TPTZ). From all these observations it appears that *Cleome speciosa* shows the greater antioxidant activity than that in other species.

Ferrous ion chelating activity (metal chelating activity)

One of the mechanisms of antioxidative action is chelation of transition metals, thus preventing catalysis of hydroperoxide decomposition and Fentontype reactions (Gordon, 1990). In the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator. The transition metal ion, Fe2+ possesses the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals (Aboul-Antioxidant properties of some Cleome species.Enein et al., 2003). The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions. The relatively mild iron(II) chelating activity of the plant extract is of great significance, because it has been proposed that the

transition metal ions contribute to the oxidative damage in neurodegenerative disorders, like Alzheimer's and Parkinson's diseases and one of the lines of treatments currently under investigation is selective low affinity binding of transition metals (Bush, 2003; Vardarajan et al., 2000). Therefore, if the plant extract also displays a mild chelating activity *in vivo* then it can be of therapeutic potential in the treatment of diseases.

Chan et al.(2009) studied the ferrous ion chelating activity from the fresh, air, and freeze-dried leaves of *Alpinia zerumbet* (11 ± 2.0 mg GAE/g), *Etlingera elatior* (17 ± 4.2 mg GAE/g), *Curcuma longa* (2.9 ± 0.1 mg GAE/g), and *Kaempferia galanga* (0.7 ± 0.1 mg GAE/g). According to Lin and Chang (2005) *Brassica oleracea* L var *italica* fresh and precooked samples have the highest ferrous ion chelating ability, at around 90%; the extract from precooked + cooked samples have a ferrous ion chelating power of 82.5%; the extract from cooked broccoli had the lowest ferrous ion chelating power, at 79.0 %. Chan et al. (2007) analyzed the metal ion-chelating properties of leaves of 26 ginger species. Their studies showed that six of the eight species clearly displayed the higher Ferrous ion chelating ability in leaves. The species were *Curcuma longa, Kaempferia galanga, Alpinia galanga, Etlingera elatior*, Zingiber spectabile, and *Etlingera maingayi*. Ferrous ion chelating values of leaves and rhizomes of *Curcuma xanthorrhiza* were comparable. At the lower extract concentration, the leaves of *Scaphochlamys kunstleri* showed lower values but, at the higher concentrations these were comparable. The EC50 values for chelating ability on ferrous ions of *Cleome* species (Fig.4) are 0.9995 ± 0.0078 for *C. viscosa;* 0.9033 ± 0.0180 for *C. simplicifolia;* 0.9079 ± 0.0311 for *C. chelidonii;* 0.8985 ± 0.0101 for *C. gynandra* and 1.0832 ± 0.0214 for *C. speciosa*.

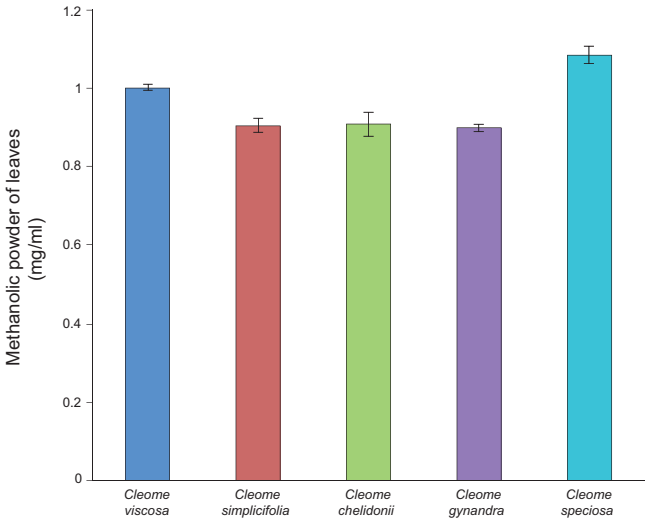

Figure 4. Ferrous ion chelating abilty in *Cleome* species (EC 50 VALUES mg/ml).

The percent inhibition activity in *Cleome speciosa* at 1000 µg/ml is 54.51±1.011% which is the highest among all *Cleome* species and that the lowest has been recorded in *Cleome chelidonii* at 200µg/ml concentration i.e. 7.07±0.508%. It is observed that inhibition percentage values go on increasing with continuous increase in concentration of methanolic plant extracts in the assay mixture (Fig 5).

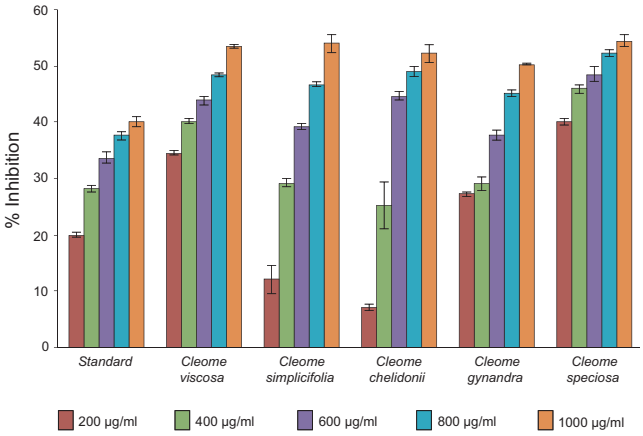

Figure 5. Ferrous ion chelating activity in *Cleome* species.

Reducing power

Reducing power assay measures the electron-donating capacity of an antioxidant (Yen and Chen, 1995). Presence of reducers causes the conversion of the Fe3+ /ferricyanide complex to the ferrous form which serves as a significant indicator of its antioxidant capacity (Yildirim et al., 2000). Bursal & Koksal (2011) evaluated the reducing power and radical scavenging activities of water and ethanol extracts from *Rhus coriaria* L. They found that the reducing power of the extracts and standard antioxidants was decreased in the order of BHA > trolox > BHT > tocopherol > water extract > ethanol extract, in presence of 30 µg/ml test sample. They observed that both reducing power and total phenolic content of water extract were higher than those of the ethanol extract. Ebrahimzadeh & Bahramian (2009) examined the antioxidant activity of *Crataegus pentaegyna* subsp. *elburensis* (CP) and noticed that methanolic and aqueous extracts exhibited a weak reducing power at 25-800 µg ml^{-1}. Deore et al. (2009) studied the reducing power of the ethanolic extracts of *Lagenaria siceraria* and noticed that the activity increases with increasing concentrations of extract. Reducing power in six Iranian olive cultivars was determined by Hajimahmoodi et al. (2008) and they found that the highest reducing power 8.331 g Vitamin E/100 g dry plant was in Mishen cultivar and that the lowest was in Conservalina. Lu et al. (2010) have reported the 53.59 ± 2.19 mg/ml as the reducing power from green tea.

In *Cleome* species, the reducing power of the extracts increases with an increase in its concentration as shown in Fig 6. Similar results were obtained by Ebrahimzadeh and Bahramian (2009) in *Crataegus pentaegyna* subsp. *elburensis*. Among all *Cleome* species, *Cleome speciosa* has shown the highest reducing power at all concentrations of its extract. 200 and 400 µg/ml concentrations of *Cleome simplicifolia* leaves have shown the lowest activity. However, at 600, 800 and 1000 µg/ml concentrations, leaf extract of *Cleome viscosa* exhibit the lowest activity. When 200 and 400 µg/ml concentrations of leaf extracts were used following order of reducing power was obtained. *Cleome speciosa* > *C. gynandra* > *C. chelidonii* > *C. viscosa* > *C. simplicifolia.* For 600 and 1000 µg/ml, *C. speciosa* > *C. gynandra* > *C. chelidonii* > *C. simplicifolia* > *C. viscosa* like pattern. However, at 800 µg/ml it was *C. speciosa* > *C. chelidonii* > *C. gynandra* > *C. simplicifolia* > *C. viscosa*. *C. speciosa* appears to have the highest reducing power among all the species studied.

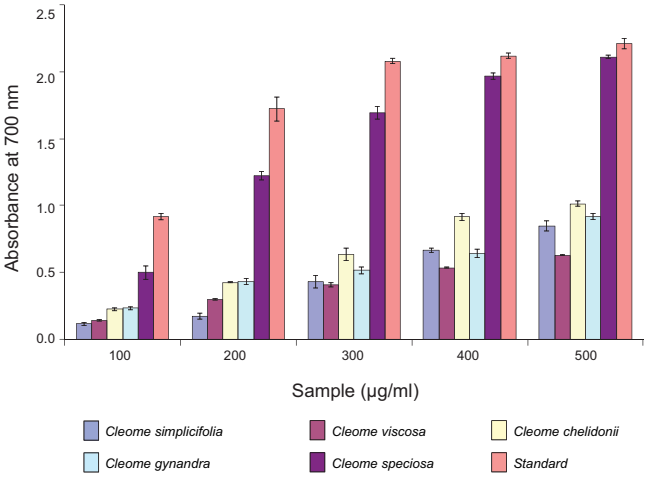

Figure 6. Reducing power in *Cleome* species.

Total antioxidant activity (TAC)

The total antioxidant activities of methanolic extracts of different *Cleome* species are recorded in Fig.7. It is evident from the results that the total antioxidant activity is the highest in *Cleome speciosa*.

Stangeland et al. (2009) have determined the total antioxidant activity in 35 Ugandan fruits and vegetables. They found that *Cleome gynandra* had this activity as 1.56 ± 0.73, that for *Amaranthus* spp. 1.00 ± 0.32, for *Solanum macrocarpon* L. 0.87 ± 0.17 and that for *Spinacia oleracea* L. as 0.98 % TAC. Antioxidant study in some edible and wound healing plants in Oman was carried out by Marwah et al. (2007). Their values for total antioxidant capacity were between 814 and 1790 mg/g of ethanol.

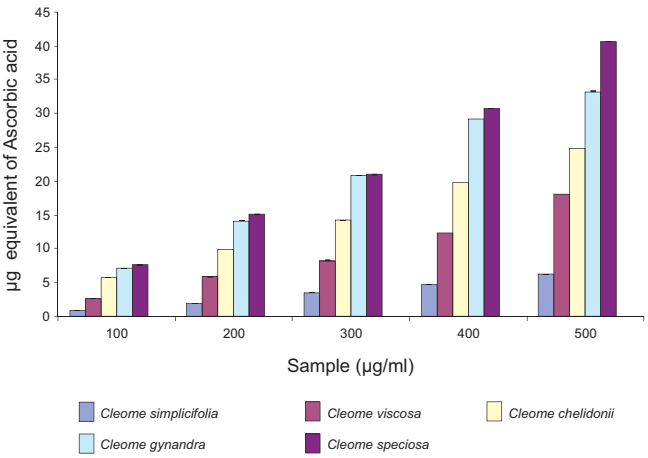

Figure 7. Total Antioxidant capacity in *Cleome* species.

Meot-Duros and Magne (2009) studied the antioxidant capacity in the leaves of *Crithmum maritimum* L. growing at two locations, during different seasons. At sand hill location it was 5.65 ± 0.26 during spring, 5.76 ± 0.17 during summer, 6.02 ± 0.70 during autumn and 4.09 ± 1.51 mg/g DW TAC during winter. At the second location, Cliffs, the antioxidant capacity was 6.78 ± 0.14 during spring, 7.04 ± 0.65 b during summer, 4.03 ± 0.59 during autumn and 5.19 ± 0.59 mg/g DW TAC during winter.

It is found that in all *Cleome* species as the concentration of leaf extract increases, the TAC activity also goes on increasing continuously. The highest TAC activity was observed in *Cleome speciosa* (for 0.2mg/ml, 7.6; for 0.4mg/ml, 15.01; for 0.6mg/ml, 20.92; for 0.8mg/ml, 30.73 and for 1mg/ml, 40.63µg equivalent of ascorbic acid). However, the lowest activity is observed in *Cleome simplicifolia* (for 0.2mg/ml, 0.92µg; for 0.4mg/ml, 1.94µg; for 0.6mg/ml, 3.41µg; for 0.8mg/ml, 4.64µg and for 1mg/ml, 6.22µg equivalent of ascorbic acid). The high level absorbance indicates the strong antioxidant capacity. As *Cleome speciosa* has shown the highest absorbance reading, it posseses the highest total antioxidant capacity.

DPPH, FRAP, TAC, Metal chelating assay and Reducing power assay mainly carried out for studying free radical scavenging activity besides phenolic radicals. In *Cleome speciosa* lowest polyphenol content observed, it may due to reaction between other biological antioxidant compounds and polyphenols as early explained by Bondet et al. (1997) that there is some reaction of DPPH occurs with certain phenolic compounds which affects level of these compounds. As per most of the assay (FRAP, TAC, Metal chelating assay and Reducing power assay) *Cleome speciosa* has shown highest antioxidant capacity. While by DPPH assay *Cleome chelidonii* has highest antioxidant power. Similarly as per DPPH, FRAP and Reducing power assay *Cleome viscosa* has

lowest antioxidant capacity but that the lowest in *Cleome simplicifolia* as per TAC, and the lowest in *Cleome chelidonii* as per Metal chelating assay. All this information indicates that all these assays help differently in scavenging free radical ions. Each assay has specificity of free radicals, therefore, all these assays are essential to study any species.

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