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Research Article

IN-VITRO ANTIOXIDANT ACTIVITY OF *CHROMOLAENA ODORATA* (L.) KING & ROBINSON

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ABSTRACT

Free radicals result in number of human degenerative diseases affecting a wide variety of physiological functions such as atherosclerosis, diabetes, ischemia/reperfusion (I/R) injury, inflammatory diseases (rheumatoid arthritis, inflammatory bowel diseases and pancreatitis), cancer, neurological diseases, hypertension. Antioxidants are an inhibitor of the process of oxidation, even at relatively small concentration and thus have diverse physiological role in the body. Antioxidant constituents of the plant material act as radical scavengers and helps in converting the radicals to less reactive species. The present study was designed to evaluate the antioxidant effects of the ethanolic extract of *Chromolaena odorata* (L.) King & Robinson. *In vitro* methods of assessment were used to determine the scavenging activity of the extract on 1,1-diphenyl-2-picryl hydrazyl (DPPH), superoxide scavenging, reducing power, nitric oxide scavenging, 2,2'azinobis(3- ethylbenzothiazoline-6-sulfonate) (ABTS), hydroxyl radical, ferric reducing antioxidant power (FRAP) and chelating ability of ethanolic extract of *Chromolaena odorata*. Generally the antioxidant activities of *Chromolaena odorata* ethanolic extract is comparable to that of butylated hydroxyanisole, mannitol, Ethylene-diaminetetraacetic acid (EDTA) and ascorbic acid to a certain extent. All the results of the *in vitro* antioxidant assays reveal potent antioxidant and free radical scavenging activity of the leaves of *Chromolaena odorata*.

Key words: *Chromolaena odorata*, antioxidant activity, DPPH.

INTRODUCTION

Free radicals are thought to play an important role in many disease such as chronic and degenerative disease including aging, coronary heart disease, inflammation, stroke, diabetes mellitus and cancer. Our body generates free radicals as by-products of burning fuel for energy with in the cells, exerting and vanishing off infections. Although oxygen is essential for aerobic forms of life, oxygen metabolites are highly toxic. Various environmental exposures such as pollution, smoke, the sun's ultraviolet light and radiation create free radicals[1].

Reactive oxygen species are considered to involve in the pathogenesis of many disease, including cancer, diabetes mellitus and atherosclerosis. Synthetic antioxidant such as butylated hydroxyl anisole (BHA) and buthylated hydroxyl tolune (BHT) are powerful. However, they are proved to be toxic to humans so that they are just industrial use[2]. Therefore, it is urgent to find natural antioxidants.

Antioxidants from natural sources play a paramount role in helping endogenous antioxidants to neutralize oxidative stress. Antioxidants inhibit or prevent oxidation of substrates and evolve to protect cells against the damage effects of reactive oxygen species (ROS), such as singlet oxygen, superoxide, hydroxy radical etc. An imbalance between antioxidants and ROS results in oxidative stress, which leads the cellular damage [3].

Chromolaena odorata(L.) King and Robinson (formerly Eupatorium odoratum) is used as a traditional medicine in Vietnam. Traditionally, fresh leaves or decoction have been used throughout Vietnam for many years as well as in other tropical countries for the treatment of leech bite, soft tissue wounds, burn wounds, skin infection and dento-alveolitis[4,5]. *Chloromalea odorata* also possess antifungal and anti-bacterial properties[6]. The aim of the present study was to evaluate the antioxidant activity, reducing power and free radical scavenging activity of the ethanolic extract of *Chromolaena odorata*.

MATERIALS AND METHODS

Sample collection and preparation

The leaves of *Chromolaena odorata* were collected from Kolli Hills in Namakkal District, Tamil Nadu, India. The plant was identified at the plant anatomy research centre. A voucher specimen of the plant has been deposited (Accession No: PARC/2012/1389).The leaves were shade dried at room temperature and then ground to a fine powder in a mechanic grinder. The powdered material was then extracted using solvent ethanol in the ratio 1:10 using Soxhlet apparatus. After extracting all coloring material, the filtrate was concentrated by evaporating in a water bath under normal pressure.

Chemicals

All chemicals used including the solvents were of analytical grade. Butylated hydroxytoluene, gallic acid, Folin-Ciocalteau reagent and ascorbic acid were purchased from Merck (Bangalore,India). All other chemicals and reagents used were of the highest commercially available purity. The solvents ethanol, methanol and sulfuric acid were purchased from Mercury Chemical Company (Tamil Nadu, India). DPPH, ABTS, trichloroaceticacid, hydrogen peroxide, potassium ferric cyanide, sodium carbonate, sodium phosphate, ammonium molybdate, ascorbic acid and gallic acid were from Aldrich (Bangalore, India).

DETERMINATION OF ANTIOXIDANT CAPACITY

DPPH Radical Quenching activity

Various concentrations of ethanolic extract of the sample (4.0 ml) were mixed with 1.0 ml of methanolic solution containing DPPH radicals, resulting in the final concentration of DPPH being 0.2 mM[7]. The mixture were shaken vigorously and left to stand for 30 minutes, and the absorbance was measured at 517 nm. Ascorbic acid was used as control. The percentage of DPPH decolorization of the sample was calculated according to the equation:

$$\% \text{ decolorization} = [1-(ABS_{sample}/ABS_{control})] \times 100$$

IC_{50}value (mg extract/ml) was the inhibitory concentration at which DPPH radicals were scavenged by 50 %. Ascorbic acid was used for comparison.

Superoxide anion radical scavenging activity

This assay was based on the reduction of nitro blue tetrazolium (NBT) in the presence of NADH and phenazinemethosulphate (PMS) under aerobic condition[8]. The 3.00 ml reaction mixture contained 50 µl of lM NBT, 150 µl of lM nicotinamide adenine dinucleotide (NADH) with or without sample and Trisbuffer (0.02 M, pH 8.0). The reaction was started by adding 15µl of lM phenazinemethosulfate (PMS) to the mixture and the absorbance change was recorded to 560 nm after 2 minutes. Percent inhibition was calculated against a control without the extract.

Reducing power

The reaction mixture contained 2.5 ml various concentrations of ethanolic extract of the sample, 2.5 ml of 1% potassium ferric cyanide and 2.5 ml of 0.2 M sodium phosphate buffer[9]. The control contained all the reagents except the sample. The mixture was incubated at 50°C for 20 minutes, and was terminated by the addition of 2.5 ml of 10% (W/V) trichloroacetic acid, followed by centrifugation at 3000 rpm for 10 minutes. 2.5 ml of the supernatant upper layer was mixed with 2.5 ml distilled water and 0.5 ml of 0.1% ferric chloride, and absorbance was measured at 700 nm against blanks that contained distilled water and phosphate buffer. Increased absorbance indicated increased reducing power of the sample. Ascorbic acid was used for comparison.

Nitric oxide radical activity

Nitric oxide radical generated from sodium nitroprusside (SNP) was measured[10]. Briefly, the reaction mixture (5.0 ml) containing sodium nitroprusside (5 mM) in phosphate-buffered saline (pH 7.3), with or without the plant extract at different concentrations, was incubated at 25°C for 3 hours. The nitric oxide radical thus generated interacted with oxygen to produce the nitrite ion which was assayed at 30 minute intervals by mixing 1.0 ml of incubation mixture with an equal amount of Griess reagent. The absorbance of the chromophore (purple azo dye) formed during the diazotisation of nitrite ions with sulphanilamide and subsequent coupling with naphthylethylenediamine dihydrochloride was measured at 546 nm.

Ferric-reducing antioxidant power (FRAP) assay

The stock solution of 10 mM 2,4,6- tripyridyl-s- triazine (TPTZ) in 40mM HCL, 20 mM $FeCl_3$. $6H_2O$ and 0.3M acetate buffer (pH 3.6) was prepared[11]. The FRAP reagent contained 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution and 25 ml acetate buffer. It was freshly prepared and warmed to 37°C. FRAP reagent (900 µl) was mixed with 90 µl water and 30 µl test ethanolic extract of the sample and standard antioxidant solution. The reaction mixture was then incubated at 37°C for 30 minutes and the absorbance was recorded at 595 nm. An intense blue color complex was formed when ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex was reduced to ferrous (Fe^{2+}) form. The absorption at 540 nm was recorded.

ABTS scavenging activity

Samples were diluted to produce 0.2 to 1.0 µg/ml. The reaction was initiated by the addition of 1.0 ml of diluted ABTS to 10 µl of different concentrations of ethanolic extract of the sample or 10 µl methanol as control[12]. The absorbance was read at 734 nm and the percentage inhibition was calculated. The inhibition was calculated according to the equation $I = A_1/A_0 \times 100$, where A_0 is the absorbance of control reaction, A_1 was the absorbance of test compound.

Hydroxy radical activity

The reaction mixture 3.0 ml contained 1.0 ml of 1.5 mM $FeSO_4$, 0.7 ml of 6 mM hydrogen peroxide, 0.3 ml of 20 mM sodium salicylate, and varying concentrations of the extract[13]. After incubation for 1 hour at 37°C, the absence of the hydroxylated salicylate complex was measured at 562 nm. The percentage scavenging effect was calculated as Scavenging activity = $[1-(A_1-A_2)/A_0] \times 100\%$, Where A_0 was absorbance of the control (without extract), A_1 was the absorbance in the presence of the extract, and A_2 was the absorbance without sodium salicylate.

Chelating activity

The reaction mixture contained 1.0 ml of various concentrations of the extract, 0.1 ml of 2 mM $FeCl_2$ and 3.7 ml methanol[14]. The control contained all the reaction reagents except the sample. The reaction was initiated by the addition of 2.0 ml of 5 mM ferrozine. After 10 minutes at room temperature, the absorbance of the mixture was determined at 562 nm against a blank. A lower absorbance of the reaction mixture indicated a higher iron chelating ability. The capacity to chelate the ferrous ion was calculated by % chelation = $[1-(ABS_{sample}/ABS_{control}] \times 100$.

Table 1. DPPH radical scavenging activity of ethanolic extract of *Chromolaena odorata*

Concentration(µg/ml)	DPPH (%)	
	Chromolaena odorata	Ascorbic acid
200	24.68±0.42	32.23±0.24
400	32.16±0.41	38.16±0.32
600	39.46±0.44	48.32±0.26
800	52.16±0.31	58.16±0.34
1000	61.78±0.36	68.08±0.46

DPPH radical scavenging activity of ethanolic extract of *Chromolaena odorata* and ascorbic acid, values are expressed as Mean ± SD (n = 3)

Table 2. Superoxide scavenging activity and reducing power by ethanolic extract of *Chromolaena odorata*

Concentration (µg/ml)	Superoxide scavenging (%)		Reducing Power (Absorbance)	
	Chromolaena odorata	Ascorbic acid	*Chromolaena odorata*	Ascorbic acid
200	19.24±0.13	22.98±0.22	0.33±0.17	0.39±0.19
400	28.42±0.18	34.01±0.28	0.42±0.23	0.55±0.24
600	41.24±0.35	45.98±0.32	0.68±0.19	0.78±0.25
800	49.48±0.33	55.63±0.43	0.81±0.28	0.89±0.29
1000	59.12±0.41	65.12±0.48	0.96±0.22	1.09±0.31

Superoxide scavenging activity and reducing power by ethanolic extract of *Chromolaena odorata* and ascorbic acid, values are expressed as Mean ± SD (n = 3)

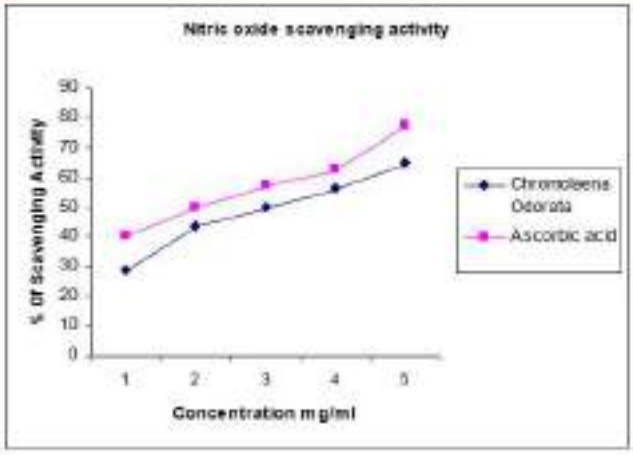


Figure 1. Nitric oxide scavenging activity of ethanolic extract of *Chromolaena odorata* and ascorbic acid
Values are expressed as mean ± standard deviation (n= 3).

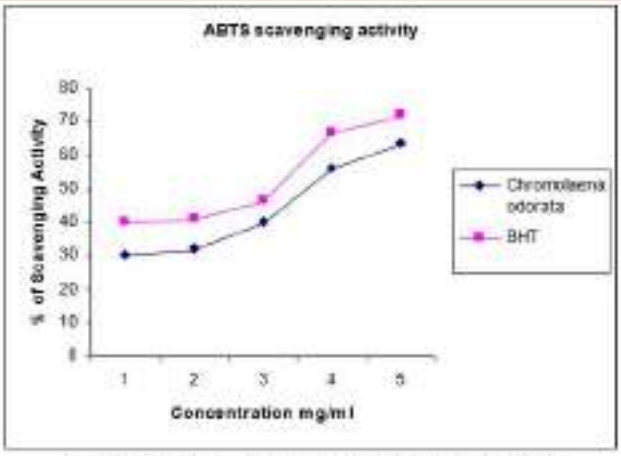


Figure 3. ABTS scavenging activity of ethanolic extract of *Chromolaena odorata* and BHT
Values are expressed as mean ± SD SEM (n =3).

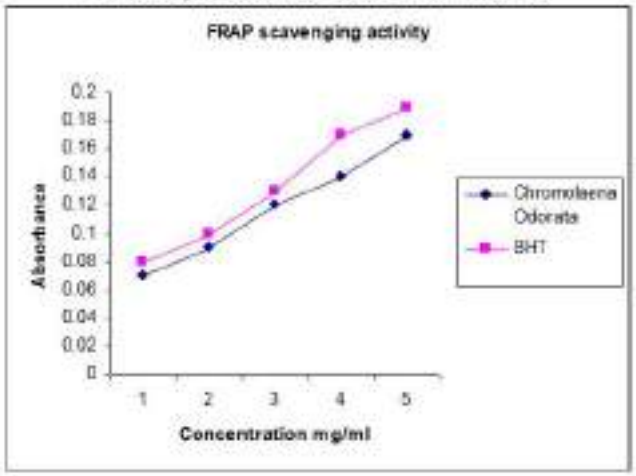


Figure 2. Ferric reducing antioxidant power of ethanolic extract of *Chromolaena odorata* and BHT
Values are expressed as mean ± standard deviation (n= 3).

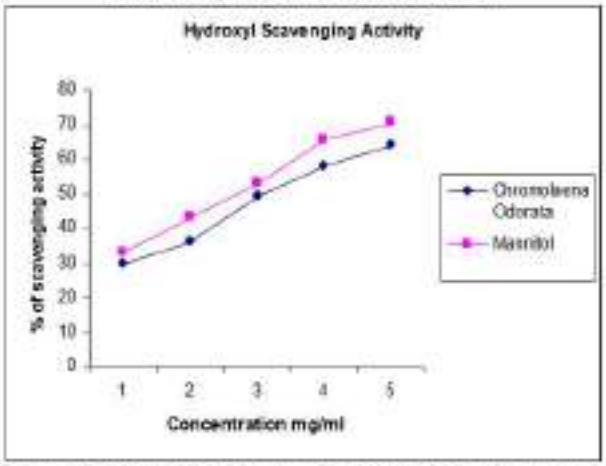


Figure 4. Hydroxyl radical scavenging activity of ethanolic extract of *Chromolaena odorata* and mannitol.
Values are expressed as mean± standard deviation (n= 3).

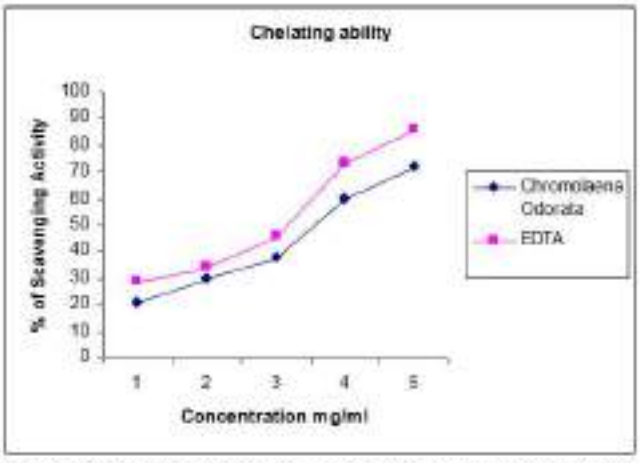


Figure 5. Chelating ability of ethanolic extract of *Chromolaena odorata* and EDTA

Values are expressed as mean ±standard deviation (n =3).

RESULTS

1, 1 Dipheny l-2-picryl-hydrazyl (DPPH) free radical scavenging activity

This method is based on scavenging of the 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) from the antioxidants, which produces a decrease in absorbance at 517 nm. The antioxidant activities of ethanolic extract of *C.odorata* and the standard ascorbic acid were 24.68-61.78% and 32.23-68.08% respectively at 200-1000µg concentrations. The table 1 shows the dose-dependent DPPH scavenging activity of *C.odorata* with an IC_{50} value of 780µg and ascorbic acid with an IC_{50} value of 600 µg.

Superoxide radical scavenging activity

The superoxide radical reduced NBT to a blue color formazon that can be measured at 560nm. At 200-1000 µg, the superoxide scavenging activity of ethanolic extract of *C.odorata* was 19.42-56.12%, and then the standard ascorbic acid value was 22.98-65.12%. The Table 2 shows the concentration-dependent radical scavenging activity, that is, percentage of inhibition increased with sample concentration.

Reducing power

Increase in the absorbance indicates increase in the antioxidant activity. In this method antioxidant compound forms a colour complex with potassium ferricyanide, trichloroacetic acid and ferric chloride, which is measured at 700nm. The ethanolic extract of *C.odorata* showed the increased absorbance in the increased concentration ranging from 200 µg to 1000 µg. Table 2. indicates the dose-response curves of the reducing power of the extracts.

Nitric oxide scavenging activity

The nitric oxide scavenging activity of the ethanolic extract of *C.odorata* were 28.78% at the minimum concentration of 200 µg, whereas the maximum activity were 64.68% at 1000 µg. Figure 1 indicates the percentage inhibition was increased with increasing concentration of the extracts. However, the activity of ascorbic acid was more pronounced than that of the extracts of *C.odorata*.

Ferric reducing antioxidant power (FRAP) assay

The ferric ability of the *C.odorata* extracts at 200-1000 µg were in the range of 0.07-0.17, then the standard ascorbic acid was 0.08-0.19% .The FRAP values for the extracts were significantly lower than those of ascorbic acid. Figure 2 shows the increased absorbance when the concentration increases.

ABTS scavenging activity

The reduction capacity of ABTS radical was determined by the decrease in its absorbance at 734nm which is induced by antioxidants. The ABTS scavenging activity of *C.odorata* extract at the concentration from 200 µg to 1000 µg were 29.92-63.34%. Ascorbic acid at a concentration of (200-1000 µg) also found to produce dose dependent inhibition of ABTS radicals was shown in Figure 3.

Hydroxyl radical scavenging

The ability of the ethanolic extracts of *C.odorata* to quench hydroxyl radicals seems to be directly related to the prevention of propagation of the process of lipid peroxidation and seems to be good scavenger of active oxygen species. The Figure 4 shows the both extracts displayed potential inhibitory effect of hydroxyl radical scavenging activity. The *C.odorata* extracts exhibited the minimum activity of 29.78% at 200 µg then the maximum activity of 64.14% at 1000 µg.

Chelating ability

The ethanolic extracts of *C.odorata* were evaluated for its chelating ability. The minimum chelating ability of the extracts were 20.68% at 200 µg, and the maximum ability were 71.96% at 1000 µg concentration. EDTA used as standard showed strong activity. Figure 5 shows the absorbance of Fe^{+}-ferrozine complex was decreased dose-dependently, that is, the activity was increased as concentration increased.

DISCUSSION

DPPH free radical scavenging activity

DPPH is a commercial oxidising radical, which can be reduced by antioxidants. The stable DPPH can be used to study the reaction kinetics of antioxidants, quantify and to compare the free radical scavenging capacities of different antioxidants[15]. In the present study, ethanolic extract of *Chromolaena odorata* showed potential free radical scavenging activity. DPPH is a stable and nitrogen centered violet colored free radical that upon reduction is converted to yellow by electron or hydrogen donating ability of the antioxidant compound found in the extract[16]. Thus an antioxidant candidate which proves promising in the DPPH antioxidant assay would provide an optimistic scaffold for prospective in vivo studies.

The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH-H by the reaction. With this method it was possible to determine the anti-radical power of an antioxidant by measuring of a decrease in the absorbance of DPPH at 517 nm[17]. Ascorbic acid is the reagent used as standard.

Superoxide radical scavenging activity

Superoxide is biologically important because it can be decomposed to form stronger oxidative species such as singlet oxygen and hydroxyl radicals[18]. The superoxide anion can be generated by illuminating a solution containing riboflavin. It is a very harmful species to cellular compounds as a precursor of more reactive species. Superoxide anion derived from dissolved oxygen by PMS–NADH coupling reaction reduces NBT in this system. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to produce the blue formazan which is measured spectrophotometrically at 560 nm and antioxidants are able to inhibit the blue NBT formation[19]. The superoxide scavenging activity of the ethanolic extract of *C.odorata* has the potential to scavenge superoxide anions. It was reported that the superoxide anion scavenging activity could be due to the action of a free hydroxyl group of phenolic compounds.

Reducing power

Reducing power is generally associated with the presence of reductones, which exert antioxidant action by breaking the free radical chain through donating a hydrogen atom[20]. In this assay, Fe3+/ferricyanide complex is reduced to the ferrous form by antioxidants and can be monitored by measuring the formation of navy blue colour at 700 nm[21].The reducing properties are generally associated with the presence of reductones[22], which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom and the activities of antioxidants have been attributed to various mechanisms such as prevention of chain initiation, decomposition of peroxides, reducing capacity and radical scavenging[23].

Nitric oxide scavenging activity

Nitric oxide formed during their reduction with oxygen with superoxide, such as NO_2, N_2O_4, N_2O_4 is very reactive[24]. These radicals are responsible for alternating the structure and functional behaviour of many cellular components. *C.odorata* extract decreases the amount of nitrite generated

from the decomposition of sodium nitroprusside in vitro. This may be due to the antioxidant principle in *C.odorata* extract, which competes with oxygen to react with nitric oxide thereby inhibiting the generation of nitrite. The plant products may have the property to counteract the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation *in vivo*[25].

Ferric-reducing antioxidant power (FRAP)

FRAP assay is based on the capability of antioxidant to reduce TPTZeFe (III) complex to the TPTZeFe (II) complex, forming a powerful blue $Fe2^{+}$eTPTZ complex with an absorption maximum at 593 nm[26]. The antioxidant properties of the plant extracts were evaluated through FRAP assays. It must also be noted that the antioxidant activities assessed are in direct relation with the polyphenolic content of the extract. Finally, a good correlation coefficient was found between the FRAP assay radical scavenging assay. The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity. A higher absorbance indicates a higher ferric reducing power[27].

ABTS radical scavenging activity

ABTS radical scavenging activity is relatively recent one, which involves a more drastic radical, chemically produced and is often used for screening complex antioxidant mixtures such as plant extracts beverages and biological fluids. The ability in both the organic and aqueous media and the stability in a wide pH range raised the interest in the use of $ABTS^{+}$ for the estimation of antioxidant activity. ABTS, a protonated radical, has characteristic absorbance maxima at 734 nm which decreases with the scavenging of the proton radicals[28]. The health promoting properties of *C.odorata* may be due to its antioxidant properties and is also attributed to its multi therapeutic characteristics. In the present study, results, *C.odorata* showed a higher antioxidant capacity. The extracts showed potent antioxidant activity in ABTS method which is comparable to the standard used.

Hydroxyl radical scavenging activity

Hydrogen peroxide itself is not very reactive, but some time it is toxic to cell because it may give rise to hydroxyl radical in the cells[24]. Hydroxyl radical scavenging capacity of *C.odorata* extract is directly related to its antioxidant activity. This method involves in vitro generation of hydroxyl radicals using Fe^{3+} /ascorbate/EDTA/H_2O_2 system using Fenton reaction. The oxygen derived hydroxyl radicals along with the added transition metal ion (Fe^{2+}) causes the degradation of deoxyribose into malondialdehyde which produces a pink chromogen with thiobarbituricacid[29]. It is clear from the result that the extracts have shown concentration dependent radical scavenging activity.

Chelating ability

An important mechanism of antioxidant activity is the ability to chelate/deactivate transition metals, which possess the ability to catalyse hydro peroxide decomposition and Fenton-type reactions. Therefore, it was considered of importance to screen the iron (II) chelating ability to the extracts[30]. In the present study, there was overwhelming evidence that the ethanolic extract of *C.odorata* had high chelating activity *in vitro*. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. Measurement of the rate of color reduction therefore allows estimation of the chelating activity of the coexisting chelator [31].

CONCLUSION

The studied medicinal plant revealed interesting antioxidant properties that could provide scientific evidence for some folk uses in the treatment of diseases related to the production of ROS and oxidative stress, but further experiments are required to explore the mechanism of action. Traditional medicine whose knowledge and practices orally transmitted over the centuries, are important approaches for discovering therapeutic molecules and compounds. Neverthless, on the basis of the results obtained, the alleged antioxidant properties might be somewhat beneficial to the antioxidant protection system against oxidative damage and the use of *Chromolaena odorata* leaf as a natural antioxidant source appears to be an alternative to synthetic antioxidant.

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