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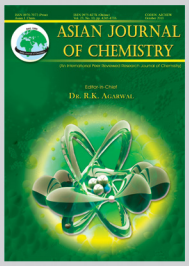

Antioxidant Activity of Ethanol Extracts from *Tridax procumbens*

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Tridax procumbens growing in subtropical areas of China as invasive plant, was investigated for total flavonoids (TF) content and antioxidant activity in the present study. Total flavonoid content was measured according to the $NaNO_2$-$Al(NO_3)_3$ method and antioxidant activity was assessed using four methods: DPPH (1,1-diphenyl-2-picryl-hydrazyl), ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), FRAP (ferric reducing antioxidant power) and TRP (the reducing power) assays. In addition, in present study, correlation analysis was made. The obtained results revealed that ethanolic extracts from *Tridax procumbens* owned better free radical scavenging activity and reducing power and showed varying degrees of efficacy in a dose-dependent manner in all the four assays. The sequence of antioxidant activity of ethanolic extracts from different parts of *Tridax procumbens* was as follows: flower > stem > the whole grass > leaf. The ethanolic extract from the whole grass of *Tridax procumbens* was added to Diaion HP-20 macroporous resin column, then the resin was washed by distilled water to get rid of impurity and M_{20}, M_{40} and M_{60} was eluted by 20, 40 and 60 % methanol individually. The sequence of antioxidant activity of these eluates was as follows: $M_{20} > M_{40} > M_{60}$, total flavonoids content and the results of antioxidant activity had significant correlation. The present study suggested that *Tridax procumbens* could be a potential rich source of natural antioxidants.

Key Words: *Tridax procumbens*, Total flavonoids, Antioxidant activity, DPPH, ABTS, FRAP, TRP.

INTRODUCTION

As the further development of molecular biology and medicine and the advancement of measuring technology, there has been an increasing understanding on the damage of free radicals and numerous studies demonstrate that there is a remarkable correlation between the excessive amount of active oxygen, free radicals and aging, cancer, cardiovascular and inflammatory disease of the body. However, synthetic antioxidants have been suspected of being responsible for liver damage and carcinogenesis. Thus, it is essential to develop and utilize effective and natural antioxidants so that they can protect the human body from free radicals and retard the progress of many chronic disease[1-3]. The researchers are looking for natural antioxidants with the characteristics of safety, high efficiency and nontoxicity, due to the possible negative effects of synthetic antioxidants.

Tridax procumbens Linn is the species of *Tridax procumbens* genus of the family compositae, is a perennials and originated from tropical areas of American, latter spread into India, South Peninsula, Indonesia and China. It is used for therapy of hepatitis, hypertension, dysuria, dysentery and hair loss. *Tridax procumbens* leaf and flower have antisepsis, antiinflammation and hemostasis functions[4,5]. In view of the medicinal values of *Tridax procumbens*, our laboratory studied its chemical constituents and some flavonoids and other compounds were isolated[6,7]. Flavonoids with phenolic hydroxyl groups, are able to provide electronic (hydrogen) to free radical lipid peroxidation (LOO), which transforms it into a more stable lipid peroxide (LOOH) and themselves become more stable phenoxy free radical or its homologue, thus interrupting the radical chain reaction, hence flavonoids are also known as absorbent of free radical. Most of them can be used for the treatment of aging, cancer, cardiovascular disease and high blood pressure, *etc.*[8,9]. However, there is not any report about the antioxidant activity on *Tridax procumbens*. Therefore, the objective of the present study was to evaluate the antioxidant activity *in vitro* of *Tridax procumbens* using four different assays respectively.

EXPERIMENTAL

Plant materials and extraction: *Tridax procumbens* was collected from Sanya City, Hainan Province, China. It was identified as the whole grass of *Tridax procumbens* Linn of *Tridax procumbens* genus of the family compositae by Shi-man Huang, Professor of Plant Taxonomy of Hainan University, the specimen were deposited in our laboratory. The dried plants

were powdered and passed through sieve No. 4 and stored the powder in airtight containers at 4 ºC until use.

The dried powder of the whole grass, leaf, flower and stem of *Tridax procumbens* (1 g) were weighed, respectively, then extracted in ultrasonic cleaner at 25 ºC, with 30 times of 70 % ethanol for three times (0.5 h each time), the extracts were concentrated into a dryness powder by the rotary evaporator and then dissolved them to volumetric flasks of 10 mL using 70 % ethanol.

The dried powder of the whole grass of *Tridax procumbens* (5 kg) was extracted with ten times of 70 % ethanol for four times at room temperature (3 days each time), the extract was concentrated into a dryness powder, then dispersed into water by ultrasonic. The water solution was added to Diaion HP-20 macroporous resin column, then the resin was washed by distilled water to get rid of impurity, then washed by 20, 40 and 60 % methanol individually and obtained M_{20}, M_{40} and M_{60}, respectively. The water eluate was abandoned and the other eluates were concentrated for using.

Infinite M200 universal microplate spectrophotometer (Swiss Tecan Company, Swiss) was used to measure the absorbance (DPPH, ABTS, FARP and TRP assays) and UV-2102 PCS UV-VIS spectrophotometer (Shanghai Unica Company Ltd., China) was used to measure the absorbance of $NaNO_2$-$Al(NO_3)_3$ method. 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), 1,1-diphenyl-2-picryl-hydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic (Trolox) are all purchased from Sigma Company (USA). Standard sample of rutin was purchased from China Pharmaceutical and Biological Products Testing Station (The batch numbers was 10080-200306). All the other chemicals used including the solvents were of analytical grade.

Quantification of flavonoids: The total flavonoids content was determined according to the $NaNO_2$-$Al(NO_3)_3$ method[10]. The standard curve was obtained using rutin concentration (X) as the abscissa axis and absorption values (Y) as the vertical axis, Y = 130.49X-0.0139, R^2=0.9991. The results were expressed as rutin equivalent (RE).

Determination of antioxidant activity

DPPH radical scavenging activity: The DPPH radical scavenging activity was evaluated according to the method of Choi *et al.*[11] with a slight modification. Briefly, 50 µL of sample solution (with various concentrations) was added to 200 µL of DPPH ethanol solution (0.2 mg/mL). After mixing gently and standing at 24 ºC for 0.5 h, the absorbance was measured at 519 nm using a microplate reader spectrophotometer $VERSA_{max}$. The DPPH free radical scavenging rate was calculated using the following formula: scavenging % = 1- (A_p-A_c)/A_{max} × 100 %. Here, A_p was the stable absorbance of DPPH ethanol solution (200 µL) plus sample solution (50 µL), A_c was the stable absorbance of 70 % ethanol solution (200 µL) plus sample solution (50 µL) and A_{max} was the stable absorbance of DPPH ethanol solution (200 µL) plus 70 % ethanol solution (50 µL).

ABTS radical scavenging activity: The ABTS radical scavenging activity was evaluated according to the method of Roberta[12] with some modifications. The stock solutions included 25 mL of ABTS solution (7 mmol/L) and 440 µL of potassium persulfate solution (140 mmol/L), then allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 mL of ABTS solution with 50 mL methanol to obtain an absorbance of 0.72 ± 0.01 units at 740 nm using a spectrophotometer. Fresh ABTS solution was prepared for each assay. 10 µL of each sample solution (with various concentrations) was added to 300 µL of the ABTS solution and standing at 24 ºC for 10 min, then the absorbance was measured at 740 nm using a microplate reader spectrophotometer $VERSA_{max}$. The ABTS free radical scavenging rate was calculated using the following formula: scavenging % = 1-(A_p-A_c) /A_{max} × 100 %. Here, A_p was the stable absorbance of the ABTS solution (300 µL) plus sample solution (10 µL), A_c was the stable absorbance of 70 % ethanol solution (300 µL) plus sample solution (10 µL) and A_{max} was the stable absorbance of the ABTS solution (300 µL) plus 70 % ethanol solution (10 µL).

Total antioxidant activity (ferric reducing antioxidant power, FRAP): The total antioxidant activity of *Tridax procumbens* were measured using FRAP assay according to the method of Gao *et al.*[13] and Benzie & Strain[14]. The reaction was carried out in a microtiter plate. 10 µL of each sample with appropriate dilution if necessary, was added to 300 µL of FRAP reagent[15] (10 parts of 0.3 mol/L sodium acetate buffer at pH 3.7, 1 part of 0.01 mol/L TPTZ solution in 40 mmol/L HCl and 1 part of 0.02 mol/L $FeCl_3 \cdot 6H_2O$ solution) and 10 µL of 70 % ethanol plus 300 µL of FRAP reagent was used as a blank, then the absorbance at 593 nm was read after 10 min. Fresh working solutions of Trolox were used for calibration and the standard curve was obtained using Trolox concentration (0.06-0.3 mg/mL) as the abscissa axis and absorbance as the vertical axis, Y = 3.7939X - 0.0435, (R^2 = 0.9997). The results were expressed as TEAC (Trolox Equivalent Antioxidant Capacity) values and TEAC value means mgTE/g DW(dry weight), briefly, TE (mg /g).

Determination of the reducing power: The reducing power (TRP) of *Tridax procumbens* was determined according to the method of Isabel CFRF[15] with a slight modification. Extract solution (500 µL), phosphate buffer (2.5 mL, 0.2 mol/L, pH 6.6) and potassium ferricyanide (2.5 mL, 1 %) were mixed and then reacted at 50 ºC for 0.5 h. Trichloroacetic acid (2.5 mL, 10 %) was added to the mixture. A volume of 2.5 mL from each of the above mixtures was mixed with 2 ml of distilled water and 0.5 mL of 0.1 % (w/v) ferric chloride in a test tube. After 10 min reaction, the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated a higher reducing power. Fresh working solutions of trolox were used for calibration and the standard curve was obtained using Trolox concentration (0.04-0.80mg /mL) as the abscissa axis and absorbance as the vertical axis, Y = 0.9129X + 0.218, (R^2= 0.9998). The total antioxidant capacity were calculated from the standard curve and expressed as TEAC values and TEAC value mean mgTE/g DW (dry weight), briefly, TE (mg/g).

Statistical analysis: All the data were presented as mean ± standard deviations of three determinations. Pearson's correlation test was used to assess correlations between data by using the SPSS system version 13.0 for Windows and the Figures were producted by using OriginPro7.5.

TABLE-1
TOTAL FLAVONOIDS CONTENT OF *Tridax procumbens* EXTRACTS

Samples	The whole grass	Stem	Leaf	Flower	M_{20}	M_{40}	M_{60}
TF (mg/g)	7.25 ± 0.03	7.16 ± 0.16	9.51 ± 0.27	2.28 ± 0.09	353 ± 15.3	343 ± 9.68	110 ± 9.01

Values in table 1 are expressed as means ± standard deviation (n = 3); TF = Total flavonoid.

RESULTS AND DISCUSSION

Total flavonoids content: Flavonoids are a class of yellow pigment and secondary metabolites and widespread in the plants, they are one of the body essential nutrients and the body cann't synthesis them by itself, but uptake them only from the outside environment. Pharmacological experiments show that flavonoids in the plants are active ingredients in the treatment of cardiovascular diseases and they have the same activity of the superoxide dismutase (SOD), the most important point is that they are low-toxicity[7]. Therefore, in the present study, TP content of *Tridax procumbens* extracts were determined and the results were shown in Table-1, from the data, we can know that the sequence of antioxidant activity of ethanolic extracts from different parts of *Tridax procumbens* was as follows: flower > stem > the whole grass > leaf, the sequence of antioxidant activity of eluates from Diaion HP-20 was as follows: $M_{20} > M_{40} > M_{60}$.

DPPH and $ABTS^+$ radical scavenging activity: DPPH and ABTS methods are commonly used for detecting the free radical scavenging activity and they have been widely applied to assess the antioxidant activity of various natural extracts[16,17]. The present study measured the free radical scavenging activity of *Tridax procumbens* extracts on DPPH and ABTS free radicals and the results were shown in Figs. 1 and 2. The Figures showed that *Tridax procumbens* extracts all had higher scavenging rate against DPPH and ABTS free radicals and the scavenging rate of the positive control and the extracts increased with the increasing concentration in a certain concentration scope, therefore, in the present study, Trolox was used as a positive control and IC_{50} value (concentration of scavenging rate at 50 %) was chosen to calculate TEAC value, in order to illustrate the scavenging capacity of extract on DPPH free radicals, TEAC value means the content of the sample equivalent to Trolox when the scavenging rate is 50 % and the free radical scavenging ability increased with increasing TEAC value. TEAC values of *Tridax procumbens* extracts were shown in Table-2, it revealed that the free radical scavenging ability of flower extract and M_{20} were the strongest and the free radical scavenging ability of leaf extract and M_{60} were the weakest in all *Tridax procumbens* extracts.

Total antioxidant activity and the reducing power: According to Yen[18] and Siddhuraju[19] that there are some connections between the reducing power and the antioxidant activity of antioxidant and the reducing power increased with increasing antioxidant activity. Therefore, the antioxidant activity can be illustrated by the reducing power. FRAP and TRP assays didn't aim at the scavenging activity of some kind of free radicals only, but reflect the total antioxidant activity of samples, so they are more suitable for evaluating the antioxidant activity of natural plant products including complex components. The present study measured the reducing power of *Tridax procumbens* extracts and the results were shown in Figs. 3 and 4. The figures showed that absorbance increased with increasing concentration in certain concentration range and the increasing of absorbance indicates an increasing reducing power. TEAC value was chosen as parameter and it expressed the content of sample equivalent to Trolox when the absorbance was 0.5. The results of FRAP and TRP assays are shown in Table-2, they were similar to the results obtained from the DPPH and ABTS assays, the reducing power of flower extract and M_{20} were the strongest and the reducing power of leaf extract and M_{60} were the weakest.

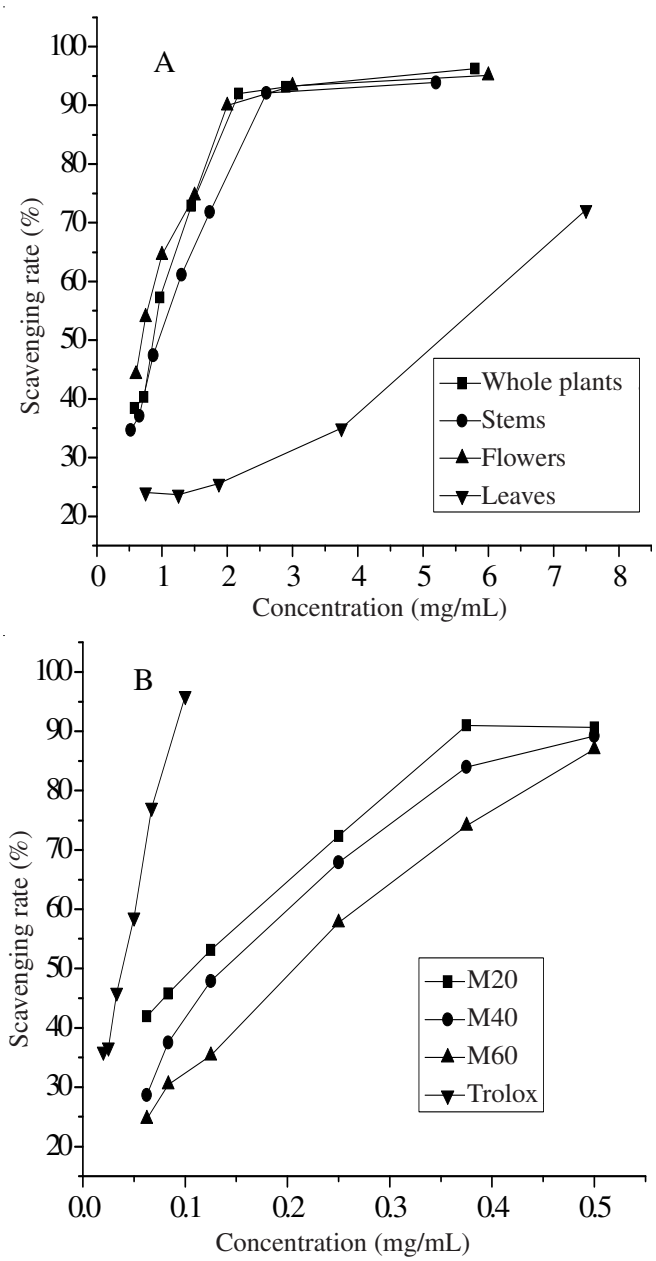
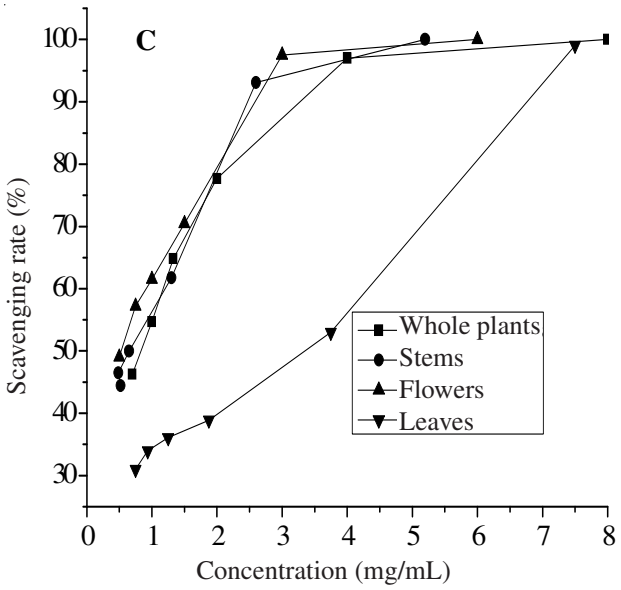


Fig. 1. Scavenging rate of the extracts on DPPH• with different concentrations. (A: different parts of *Tridax procumbens*; B: different eluates from Diaion HP-20)



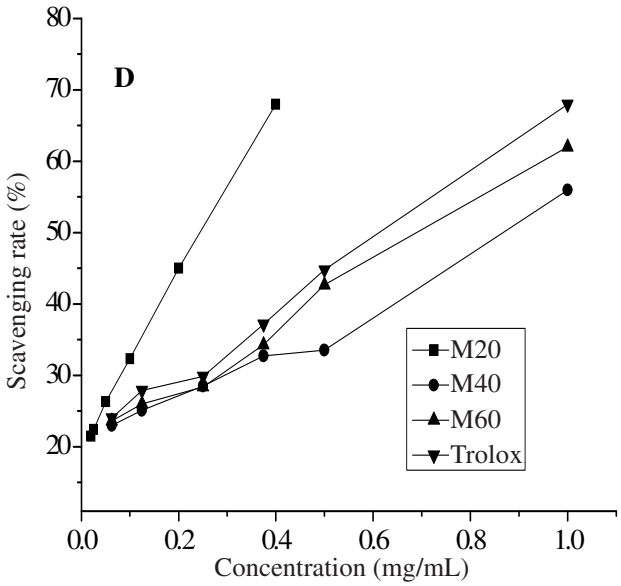
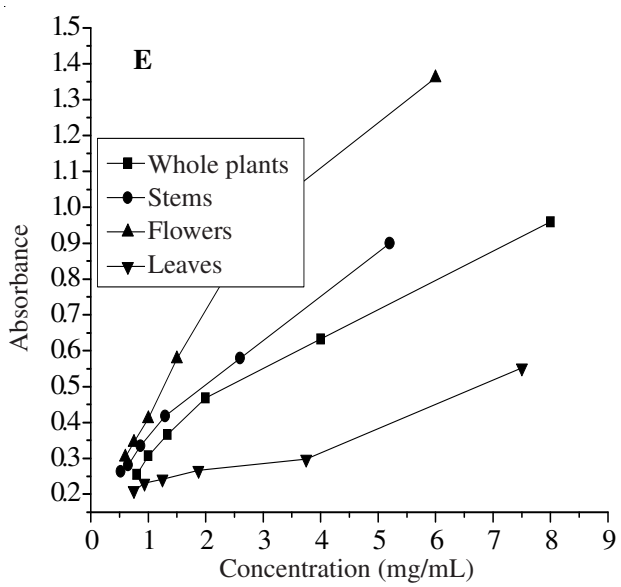


Fig. 2. Scavenging rate of the extracts on $ABTS^{+\bullet}$ with different concentrations. (C: different parts of *Tridax procumbens*, D: different eluates from Diaion HP-20)



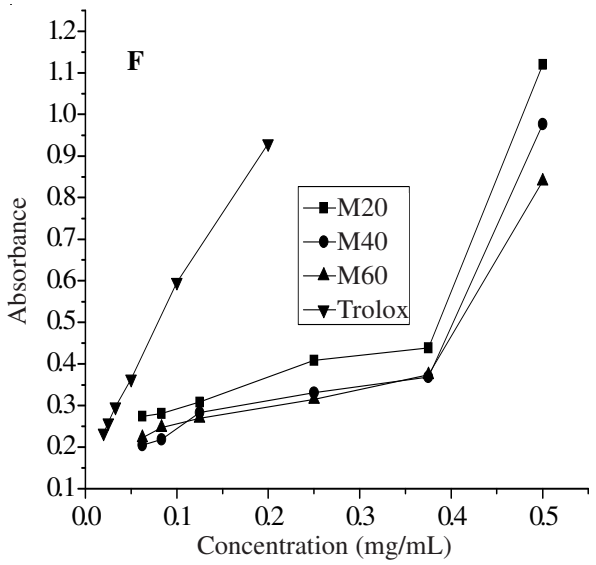
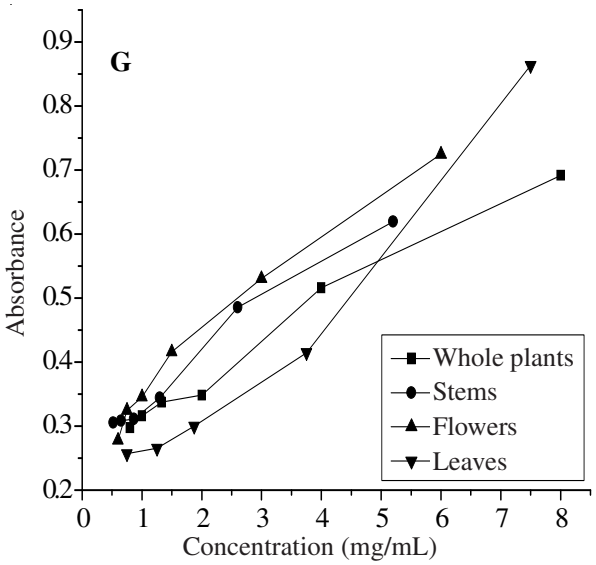


Fig. 3. Total antioxidant activity of the extracts with different concentrations. (E: different parts of *Tridax procumbens*, F: different eluates from Diaion HP-20)



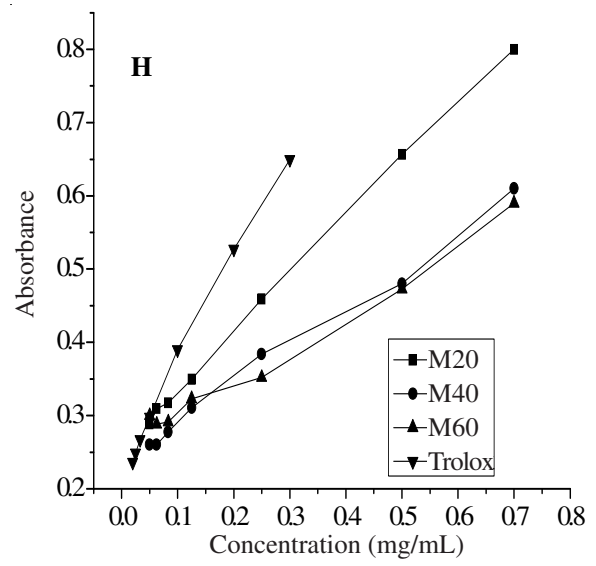


Fig. 4. Reducing power of the extracts with different concentrations (G: different parts of *Tridax procumbens*, H: different eluates from Diaion HP-20)

TABLE-2
TEAC VALUES OF *Tridax procumbens* EXTRACTS AND DIFFERENT ELUATES FROM DIAION HP-20

	Sample						
	Whole grass	Stem	Leaf	Flower	M_{20}	M_{40}	M_{60}
FRAP TEAC (mg/g)	1.920 ± 0.227	2.077 ± 0.839	0.881 ± 0.012	3.665 ± 0.259	203.526 ± 19.547	195.761 ± 19.874	192.875 ± 26.149
The reducing power TEAC (mg/g)	3.602 ± 0.351	2.440 ± 0.017	3.011 ± 0.782	4.148 ± 0.014	592.715 ± 12.304	339.015 ± 15.634	327.839 ± 24.154
DPPH TEAC (mg/g)	2.593 ± 0.077	2.099 ± 0.129	0.545 ± 0.062	3.333 ± 0.037	355.140 ± 0.012	275.362 ± 0.009	185.366 ± 0.011
ABTS TEAC (mg/g)	15.532 ± 0.041	18.739 ± 0.073	5.357 ± 0.035	27.068 ± 0.044	393.442 ± 0.052	349.854 ± 0.131	279.395 ± 0.089

Values in table 2 are expressed as means ± standard deviation (n = 3)

TABLE-3
RELATIVITY ANALYSIS AMONG THE TF CONTENT AND RESULTS OF FOUR METHODS OF ANTIOXIDANT ACTIVITY

	TF content	DPPH (TEAC)	ABTS (TEAC)	FRAP (TEAC)	TRP (TEAC)
Total flavonoid content	1	0.880**	0.911**	0.966**	0.940**
DPPH(TEAC)		1	0.942**	0.956**	0.987**
ABTS(TEAC)			1	0.984**	0.970**
FRAP(TEAC)				1	0.990**
TRP(TEAC)					1

** Correlation is significant at the 0.01 level (2-tailed).

Relativity analysis between the total flavonoids content and the results of four assays: Here, the relativity analysis between the total flavonoids content and the results of four assays was made and the results were shown in Table-3. From Table-3, we can found that the total flavonoids content and outcomes of four antioxidant activity assays had very significant relativity. It indicated that flavonoids are the main components responsible for antioxidant behaviour of *Tridax procumbens* and there were very high correlation between the measuring results of these four methods, so they are all available for the determination of antioxidant activity of *Tridax procumbens*.

Conclusion

We investigated the antioxidant activity of *Tridax procumbens in vitro*, using four methods: DPPH, ABTS, FRAP and TRP assays at the same time. The present study indicated that *Tridax procumbens* extracts all had strong scavenging activity against DPPH and ABTS free radicals and strong reducing power on Fe^{3+}. The antioxidant activity of *Tridax procumbens* owed to flavonoids compounds. The sequence of antioxidant activity of the ethanolic extracts from different parts of *Tridax procumbens* was as follows: flower > stem > the whole grass > leaf, the sequence of antioxidant activity of different eluates from Diaion HP-20 was as follows: M_{20} > M_{40} > M_{60}. *Tridax procumbens* can be utilized as an effective and safe antioxidant source.

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