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Measurement of total phenolic content and antioxidant activity of aerial parts of medicinal plant *Coronopus didymus*

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ABSTRACT

Objective: To evaluate the total phenolic content and compare the antioxidant activity of various solvent extracts and fractions from the aerial parts of *Coronopus didymus* through various assays.

Methods: Total phenolic content was determined using the Folin-Ciocalteu assay and the *in vitro* antioxidant activity of a number of different extracts was investigated in a dose-dependent manner with three different methods: the 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and ferric reducing antioxidant power (FRAP) assays. A flavone was isolated from the most active ethanolic extract with high antioxidant activity using size exclusion chromatography. IC_{50} values were calculated for the DPPH and ABTS methods. The FRAP activity was assessed in terms of μM Fe (II) equivalent.

Results: The phenolic content was found to be highest in the ethanol extract (CDA Et; 47.8 mM GAE) and the lowest in the dichloromethane extract (CDA DCM; 3.13 mM GAE). The ethanol extract showed high radical scavenging activity towards DPPH and ABTS radicals with IC_{50} values of (7.80×10^2) and (4.32×10^2) μg/mL, respectively. The most active ethanol extract had a FRAP value of 1921.7 μM Fe (II) equivalent. The isolated flavone F10C (5,7,4′-trihydroxy-3′-methoxy flavone) was far more effective for scavenging free radicals in the DPPH and ABTS assays with IC_{50} of 43.8 and 0.08 μg/mL, than the standard trolox, with IC_{50} values of 97.5 and 21.1 μg/mL, respectively. In addition, the flavone F10C and the standard ascorbic acid had FRAP values of 1621.7 and 16 038.0 μM Fe (II) equivalents, respectively.

Conclusions: The total phenolic content of extracts in decreasing order is ethanol extract (CDA Et) > acetone extract (CDA ACE) > phenolic extract (CDA MW) > *n*-hexane extract (CDA nHX)> chloroform extract (CDA CHL) > dichloromethane extract (CDA DCM). The ordering of extracts in terms of antioxidant activity from highest to lowest is CDA Et > CDA MW > CDA DCM > CDA CHL > CDA ACE > CDA nHX in DPPH, ABTS and FRAP assays. A significant relationship is found between antioxidant potential and total phenolic content, suggesting that phenolic compounds are the major contributors to the antioxidant activity of *C. didymus*.

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1. Introduction

Plants are a rich source of natural bioactive compounds such as secondary metabolites and antioxidants [1]. Phenolic compounds are the most abundant secondary metabolites in plants, playing a key role in pigmentation, growth and reproduction of the plant, together with resistance to pathogens and predators. This is largely due to their phytoalexin properties and potent astringency [2,3]. They have been shown to provide anti-allergic, anti-inflammatory, antioxidant, hepatoprotective, antiviral, and anticarcinogenic activities [4,5]. However, probably most interest has been devoted to their antioxidant activity, their ability to reduce free radical formation and to scavenge free radicals *in vivo* [6].

The use of natural antioxidants is a field of growing interest, especially in food science and complementary medicines, because some synthetic antioxidants are harmful to human health. The evaluation of antioxidant potential is, however, a crucial issue, because plants contain two main types of antioxidants, polar (phenolics) and non-polar (vitamin E), and there is no single method suitable for assessment of both types. Secondly complex composition of plant extracts can lead to contradictory results if the antioxidant activity is evaluated by a single method. So, at least two methods for evaluating antioxidant activity are therefore recommended. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging assays are the commonly used methods for evaluation of proton donating antioxidants, like phenolic compounds, from plants. Each method has its own advantage, for example, the most commonly used DPPH method is sensitive and requires little sample material while the ferric reducing antioxidant power (FRAP) method is fast, easy to handle, with highly reproducible results.

Coronopus didymus L. (*C. didymus*; lesser swinecress) is an important member of Brassicaceae family, often used traditionally in Brazil, especially in southeast region, to suppress fever, combat pain and inflammations [7]. It has also been reported to be used as an expectorant and blood purifier and, in addition there have been reports of anti-malarial and anti-cancer properties [8]. Previously we reported the identification of 68 volatile compounds from the aerial parts and roots of this plant using Gas Chromatography-Mass Spectrometry [9]. Recently we documented the bioassay-guided isolation of cytotoxic flavonoids from the aerial parts of *C. didymus* [10]. In continuation of our exploration on this plant, here we measured the total phenolic content and antioxidant activity from different solvent extracts and fractions. Previously different fractions of the whole plant aqueous extract of *C. didymus* L., and MeOH−$CHCl_3$ fractions, were evaluated for free radical scavenging ability by DPPH and ABTS assays [11]. The present study was designed to determine the total phenolic content and measure the antioxidant activity of the aerial parts of this plant using three different antioxidant methods *i.e.*, DPPH, ABTS, and FRAP assays. For assessment of antioxidant potential of extracts, the use of different methods is necessary. The three antioxidant methods have been used in this study which could be divided into two groups depending on the oxidizing reagent. Two methods use organic radical producers (DPPH, ABTS) and one method uses metal ions for oxidation (FRAP). In order to isolate antioxidants with diverse structural features and to reap the full benefits of the plant, solvents with a wide range of polarities (from non-polar to polar) were used for extraction in this study. The most active ethanol extract was further fractionated by size exclusion chromatography (SEC). The antioxidant activity of the extracts, fractions and isolated compound from the aerial parts of *C. didymus* was also reported here for the first time using a FRAP assay.

2. Materials and methods

2.1. Chemicals and instrumentation

All chemicals used were of analytical grade or higher. Gallic acid, DPPH, ABTS, potassium persulfate and 2,4,6-*tris*(2-pyridyl)-*s*-triazine (TPTZ) were purchased from Sigma–Aldrich (Gillingham, UK). Whatman® cellulose chromatography papers 1 Chr sheets, (20 × 20) cm (GE Healthcare Life Sciences, UK) were used for paper chromatography. Sephadex LH-20 (Sigma Life Science, Sweden) was used for size exclusion chromatography. UV–Vis. absorption were measured on a UV–Vis. Spectrophotometer (UV–Vis. Cary 4000, Agilent, UK) controlled by Agilent Scan software. Extracts were ultrasonicated using an ULTRASONIC LC 30 H (Elma, Germany) and concentrated using a rotary evaporator (BÜCHI Rotavapor R-200, Switzerland).

2.2. Plant material

Aerial parts of the plant were collected from wild growing areas of Sector I-8/1 Islamabad, Pakistan, in spring 2015 and dried at room temperature. The plant was authenticated by Dr. Mushtaq Ahmad, Department of Plant Sciences, Quaid-i-Azam University, Islamabad, Pakistan. A voucher specimen (No. 74) of the plant has been deposited at the Herbarium of the same Department.

2.3. Extraction and isolation

Five metabolite extractions were made separately from the dried aerial parts of *C. didymus* (70 g) by maceration at room temperature for 96 h using *n*-hexane, chloroform, acetone, dichloromethane and ethanol (1 L). All the extracts were ultrasonicated for 30 min, filtered and concentrated *in vacuo* at 45 °C. The ethanolic extract (13.2 g) was dissolved in MeOH−H_2O (7:3, v/v) and filtered to provide a phenolic extract (CDA MW, 4.7 g). The latter was subjected to SEC using a column (90 cm × 2.2 cm) packed with slurry of Sephadex LH-20. The column was eluted with methanol-water in a ratio of 7:3 (v/v), at a flow rate of 1 mL/min to yield ten fractions (F1−F10) as shown in Figure 1.

All the fractions were concentrated *in vacuo* and analysed by three different antioxidant methods. Yellow precipitates (F10C, 7 mg) were obtained from fraction F10 by SEC. Ethanol extract and its fractions were also analysed by paper chromatography (PC) using 15% acetic acid and chromatograms were visualized under UV light (365 nm).

2.4. Determination of total phenolic content (TPC)

The total phenolic content of the extracts was determined using Folin-Ciocalteu assay [12]. The Folin-Ciocalteu method is an electron transfer based assay, and gives reducing capacity

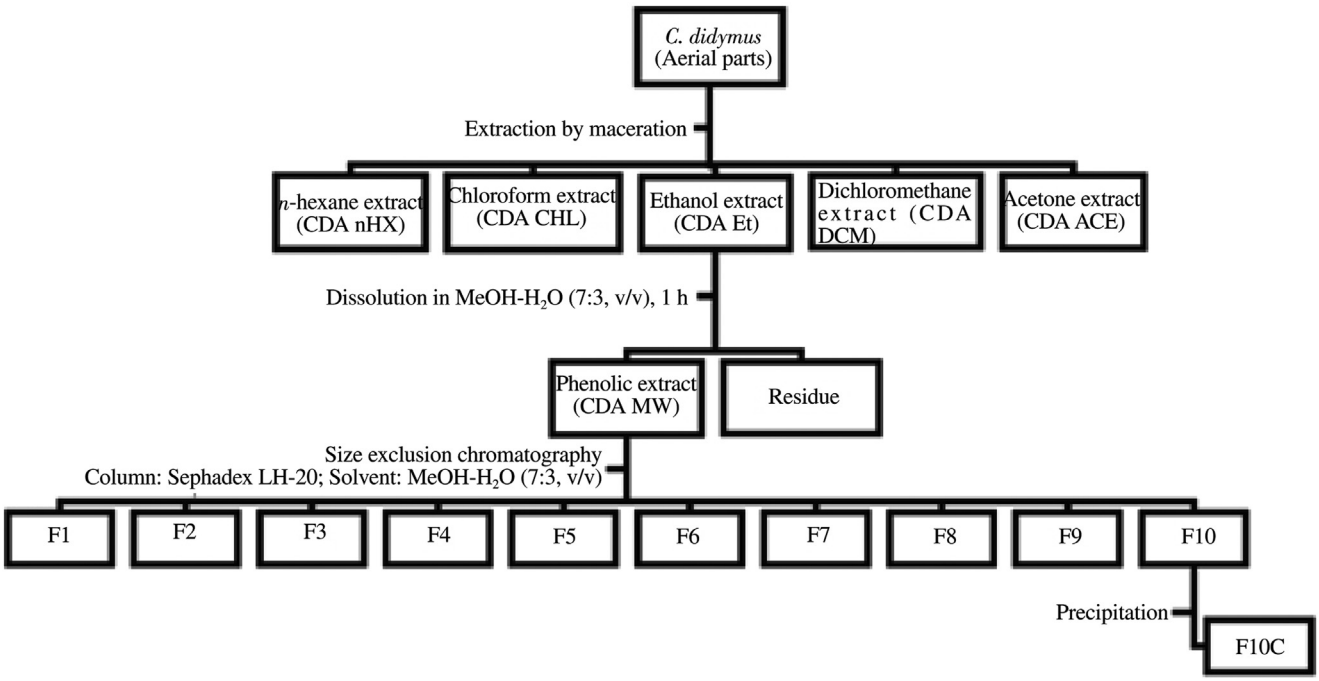

Figure 1. Flow chart showing the extraction process and bioassay-guided fractionation of ethanol extract from the aerial parts of *C. didymus*.

which is expressed as phenolic content. Total phenolic content of plant extracts and their yield depends on the solvent selected for extraction. The external calibration was done using different concentrations of gallic acid *i.e.*, 0.00, 0.25, 0.50, 0.75 and 1 mM. In brief, 200 µL of extracts (10 mg/mL) and 2.0 mL of solution A (mix 10 mL of 2% Na_2CO_3 with 0.1 mL of $CuSO_4$ and 0.1 mL of sodium and potassium tartrate) were mixed and after 4 min, 0.4 mL of 0.5 M sodium hydroxide was added. After 10 min 0.2 mL of Folin-Ciocalteu reagent (1:1 v/v with water) was added. The solution was left for 30 min and its absorbance was measured with a UV–Vis. spectrophotometer at 750 nm. The total phenolic content was calculated as mM gallic acid equivalent (mM GAE) by using gallic acid calibration curve.

2.5. Antioxidant activity

Three different chemical methods namely DPPH, ABTS and FRAP assays were used for evaluating the antioxidant activity of different extracts, fractions, and isolated compound. Stock solutions of crude extracts, each fraction from the ethanol extract and isolated compound (see Section 2.3), were prepared separately in methanol at 10 mg/mL concentration from dry weight. These stock solutions were diluted in methanol to provide four different concentrations; 50, 100, 200 and 400 µg/mL. Gallic acid, ascorbic acid and trolox standard stock solutions were also prepared in methanol at 10 mg/mL concentration and were diluted in methanol to provide four different concentrations; 50, 100, 200 and 400 µg/mL.

2.5.1. DPPH radical scavenging assay

The DPPH radical scavenging assay was carried out according to the method, reported by Öztürk *et al.* with some modifications [13]. Briefly, 0.4 mL of plant extracts, fractions, isolated compound, antioxidant standards gallic acid and trolox (50–400 µg/mL) were mixed with 3.6 mL methanolic solution of DPPH (0.1 mM). An equal amount of methanol (0.4 mL) was used as a blank (control) with 3.6 mL of DPPH solution. All the samples were prepared in triplicate, vortexed for 1 min and incubated in dark for 30 min at 37 °C. The decrease in absorbance of each sample was measured against methanol as blank on UV–Visible spectrophotometer at 517 nm. Percentage DPPH inhibition was calculated using the formula;

$$\text{DPPH inhibition}(\%) = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

The results were reported as IC_{50} value, a lower IC_{50} value represents a stronger DPPH scavenging capacity.

The DPPH assay is a free radical method based on the radical scavenging activity of antioxidants (AH) towards the purple coloured $DPPH^{\cdot}$ in MeOH. The free radical $DPPH^{\cdot}$ was reduced to the corresponding stable diamagnetic molecule hydrazine (yellow coloured) when it reacted with hydrogen donors (AH, antioxidants with acidic hydrogens) [14]. Antioxidants could be polar or non-polar in nature and they can act as radical scavenger by electron donating mechanism or by hydrogen donating mechanism. Therefore, two different control antioxidants (gallic acid and trolox) were used in this study.

2.5.2. ABTS radical scavenging assay

ABTS radical scavenging assay was carried out according to the method reported by Re *et al.* [15]. The ABTS radical cation ($ABTS^{\cdot+}$) was generated by mixing the following solutions; 7.0 mM ABTS solution in H_2O (Solution a) and 2.45 mM potassium persulfate ($K_2S_2O_8$) solution in H_2O (Solution b) in ratio of 1:1 (v/v). The reagent was kept in darkness at room temperature for 16 h to complete the reaction after which this solution was diluted with ethanol to get the ABTS working solution having absorbance of 0.70. For the assay, 100 µL of plant extracts, fractions, isolated compound and standard trolox (50–400 µg/mL) were mixed with 2.9 mL of ABTS working solution. An equal amount of ethanol (100 µL) was used as a blank (control). All the samples were prepared in

triplicate and vortexed for 1 min. After 6 min incubation, the decrease in absorbance of each sample was measured against ethanol as blank on UV–Visible spectrophotometer at 734 nm. Percentage ABTS inhibition was calculated using the formula;

$$\text{ABTS inhibition}(\%) = \frac{A_{\text{Control}} - A_{\text{Sample}}}{A_{\text{Control}}} \times 100$$

The results were reported as IC_{50} value, a lower IC_{50} value represents a stronger ABTS scavenging capacity.

2.5.3. Ferric reducing antioxidant power assay

The FRAP assay was conducted according to the method reported by Benzie and Strain [16]. FRAP reagent was prepared freshly by mixing three solutions a, b and c, 300 mM sodium acetate buffer, pH = 3.6 (solution a), 10 mM TPTZ solution in 40 mM HCl solution (b) and 20 mM ferric chloride ($FeCl_3$) solution (c) in proportions of 10:1:1 (v/v/v). The reagent was kept in darkness for 30 min to complete the reaction. For the assay, 0.1 mL of plant extracts, fractions, isolated compound, positive control ascorbic acid (50, 100, 200 and 400 μg/mL) and $FeSO_4$ (329.16, 658.3, 1316.6 and 2633.3 μM) were mixed with 2.9 mL of FRAP reagent separately. An equal amount of DMSO (0.1 mL) was used as a blank (control). All the samples were prepared in triplicate and vortexed for 1 min and incubated in dark for 30 min at 37 °C. The increase in absorbance of reaction mixture was measured for each sample on UV–Visible spectrophotometer at 593 nm. The results were compared with ascorbic acid as positive control and $FeSO_4$ was used for calibration. FRAP activity was calculated as ferrous equivalent (FE) in μM.

2.5.4. Statistical analysis

All data was presented as the mean of three separate experiments and error bars are displayed with standard error. The dose–response data was best fitted to a straight line after logarithmic-transformation of the X-axis. The dose response curves were plotted between percentage of scavenging and natural log (ln) of concentrations using SigmaPlot professional software Version 13.0. The IC_{50} value was determined from linear regression analysis using Microsoft excel with its Data Analysis add-in. IC_{50} values $\leq$ 500 μg/mL were considered active.

3. Results

3.1. Total phenolic content

As a result among all the extracts the highest extraction yield (13.2 g) was obtained from the ethanol extract of the aerial parts (CDA Et), followed by acetone (CDA ACE, 5.0 g), *n*-hexane (CDA nHX, 3.9 g) and dichloromethane (CDA DCM, 3.5 g) while the chloroform extract (CDA CHL) yielded the lowest (2.5 g). The total phenolic content of these extracts from *C. didymus* were determined using the Folin-Ciocalteu assay by constructing a standard curve with gallic acid (GA) taking into consideration the relationship between absorbance and concentration. The calibration curve generated from the analysis of the standard (gallic acid) was linear (Figure 2) with $y = 0.0384x + 0.0916$; $R^2 = 0.992$.

Using the equation obtained from calibration curve, the ethanol extract (CDA Et) showed the highest phenolic content

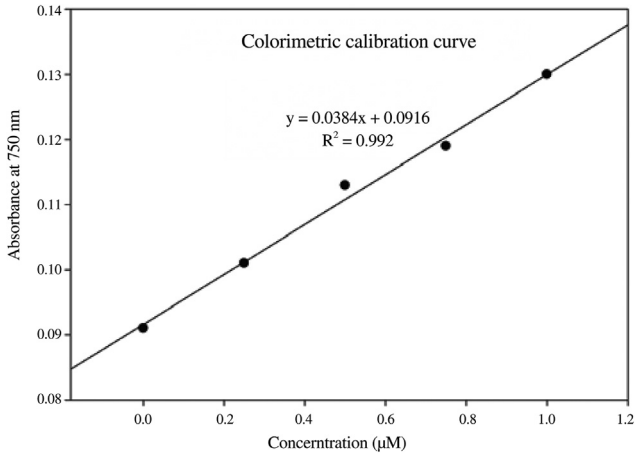

Figure 2. Gallic acid standard calibration curve for the quantification of total phenolic content.

(47.8 mM GAE) followed by acetone (38.6 mM GAE) and *n*-hexane (27.2 mM GAE) while chloroform and dichloromethane extracts showed the lowest contents (Table 1). Among the various extracts studied, variation in total phenolic content was large, up to 15 fold, ranged from 47.8 (CDA Et) to 3.13 mM GAE (CDA DCM). This result suggested that the phenolic compounds were best extracted via ethanol from *C. didymus*. So the ethanol extract was further fractionated by SEC to provide ten fractions.

3.2. DPPH free radical scavenging activity

Percentage scavenging effect of DPPH radicals versus concentration of extracts, fractions and standards (ln conc. μg/mL) were plotted (Figure 3). It was found that the percentage of DPPH radical scavenging activity of all the extracts was dose dependent as illustrated in Figure 3a. At 100 μg/mL concentration, ethanolic extract showed 32.2% DPPH scavenging (Figure 3a), while gallic acid and trolox used as standards showed 86.4% and 45.6% DPPH scavenging activity (Figure 3d). The DPPH free radical scavenging activity of the ethanol extract of *C. didymus* was significant with an IC_{50} value of (7.80×10^2) μg/mL, with high TPC value (47.8 mM GAE) among all the extracts (Table 1), so this extract was selected for further fractionation.

All the ten fractions obtained from the ethanol extract exhibited varying degrees of DPPH scavenging capacity (Figure 3b and c). At all concentrations (50–400 μg/mL) the five

Table 1

The total phenolic content (mM GA equivalent) and antioxidant activity from the analysis of a number of different solvent extracts of *C. didymus* by DPPH, ABTS [IC_{50} (μg/mL)] and FRAP [μM Fe (II) equivalent] methods.

Crude extract	Total phenolic content	DPPH	ABTS	FRAP
CDA Et	47.80	$7.80 \times 10^2 \pm 2.89$	$4.32 \times 10^2 \pm 6.59$	1921.7
CDA MW	29.80	$2.95 \times 10^3 \pm 2.59$	$1.12 \times 10^3 \pm 6.37$	1538.3
CDA nHX	27.20	$8.53 \times 10^6 \pm 2.23$	$1.33 \times 10^7 \pm 1.64$	1638.3
CDA CHL	3.34	$2.16 \times 10^6 \pm 2.09$	$2.05 \times 10^4 \pm 4.29$	1921.7
CDA ACE	38.60	$6.61 \times 10^7 \pm 1.17$	$7.16 \times 10^4 \pm 1.13$	1855.0
CDA DCM	3.13	$9.46 \times 10^5 \pm 1.27$	$8.60 \times 10^3 \pm 0.37$	1921.7

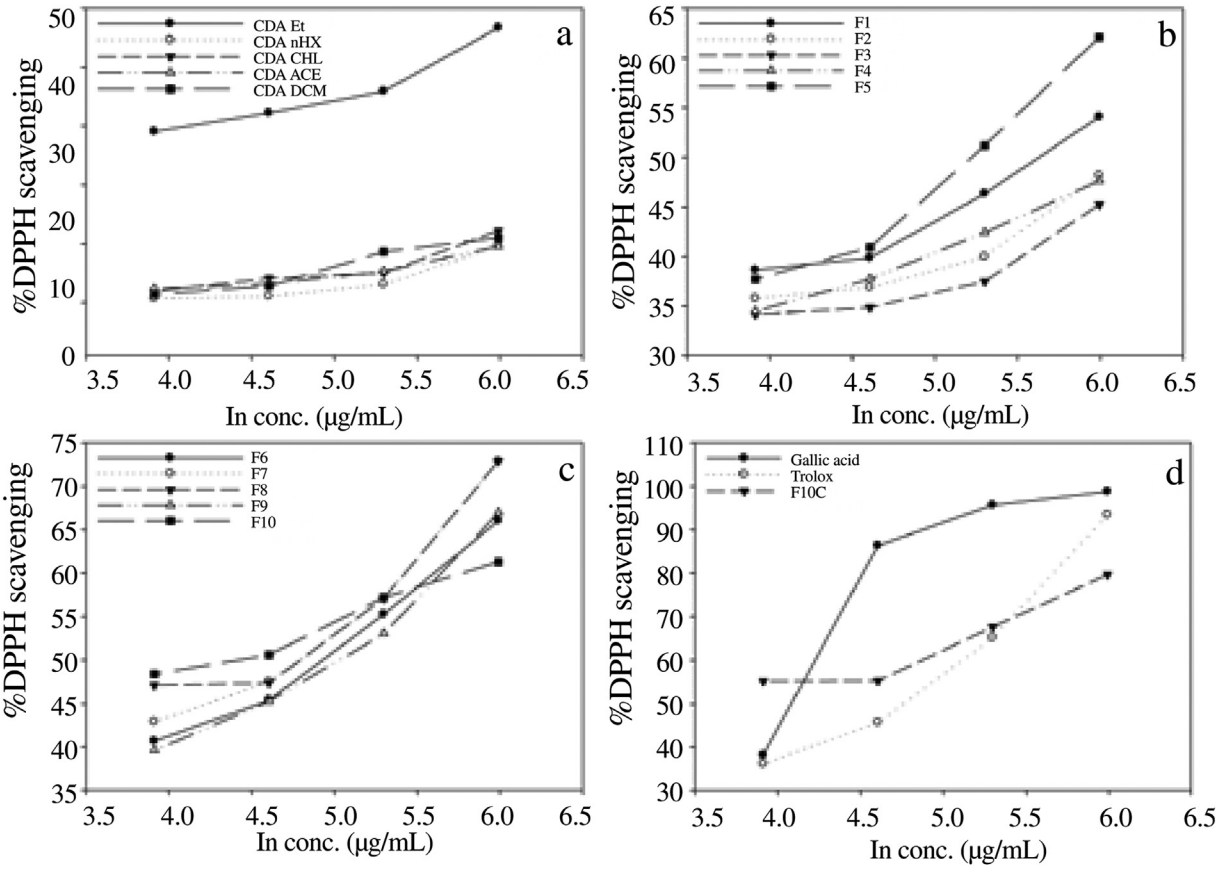

Figure 3. The percentage DPPH scavenging activity of a) various solvent extracts, b) fractions F1−F5, c) fractions F6−F10 and d) standards and isolated compound.

fractions F6−F10 showed stronger DPPH radical scavenging activity than fractions F1−F5. Among these five active fractions, the activity of the fractions towards the DPPH radical increased in the order F9 < F6 < F7 < F8 < F10, which suggested that the elution process using methanol-water via SEC also has a strong concentrating effect for radical-scavenging components in the fractions F6−F10. Collective PC analysis of these fractions revealed that flavonoids are present in the fractions F10, F9, F8 and F7. Among the various fractions, the scavenging effect of fractions F7 (47.5%), F8 (47.3%) and F10 (50.6%) (Figure 3c), at 100 µg/mL concentration were higher than the trolox (45.6%) (Figure 3d), indicating their ability to act as scavengers at low concentrations. The calculated IC_{50} values for all the fractions against DPPH radicals were given in Table 2. On the whole, fraction F10 obtained from SEC exerted the strongest DPPH radical scavenging activity among all the other fractions and crude extracts with an IC_{50} value of 72.2 µg/mL (Table 2). Fractions F8 and F7 had lower IC_{50} values compared to fractions F9 and F6, respectively.

In the DPPH assay, the isolated flavone (F10C) at 50 µg/mL concentration, showed 55.1% (Figure 3d) while trolox used as a standard antioxidant showed 36.1% scavenging activity. Interestingly F10C (5,7,4′-trihydroxy-3′-methoxy flavone) with lowest IC_{50} value of 43.8 µg/mL (Table 2) was far more effective than the trolox (IC_{50} 97.5 µg/mL).

3.3. ABTS radical scavenging assay

A dose–response relationship was found in the ABTS radical scavenging assay (Figure 4a); the activity increased as the concentration increased for each extract. In the ABTS assay, the ethanol extract at 400 µg/mL concentration, showed 53.5% (Figure 4a) while trolox used as a standard antioxidant showed 66% scavenging activity (Figure 4d). The IC_{50} values for all the extracts against ABTS radicals are given in Table 1. The ethanolic extract showed significantly stronger ABTS scavenging potency [IC_{50} (4.32×10^2) µg/mL] than that of all other extracts of plant, which was even higher than the reported DPPH scavenging activity [IC_{50} (7.80×10^2) µg/mL]. It was observed that some of the components extracted with ethanol were strong

Table 2
The antioxidant activity of fractions and isolated compound of ethanolic extract of *C. didymus* and standards by DPPH, ABTS [IC_{50} (µg/mL)] and FRAP [µM Fe (II) equivalent] methods.

Fractions and standards		DPPH	ABTS	FRAP
Fractions	F1	281.40 ± 2.40	327.00 ± 1.08	1538.3
	F2	757.40 ± 2.53	1699.30 ± 4.15	1555.0
	F3	1450.90 ± 2.55	2158.10 ± 2.44	1571.7
	F4	620.70 ± 0.69	289.70 ± 6.90	1388.3
	F5	165.60 ± 2.92	139.10 ± 7.91	2005.0
	F6	121.50 ± 2.24	68.50 ± 4.75	1871.7
	F7	98.40 ± 3.95	45.70 ± 3.14	1938.3
	F8	86.40 ± 5.51	83.50 ± 5.08	2021.7
	F9	127.70 ± 3.00	31.10 ± 7.58	1538.3
	F10	72.20 ± 3.65	46.50 ± 6.23	1638.3
	F10C	43.80 ± 4.65	0.08 ± 0.15	1621.7
Standards	Gallic acid	4.80 ± 1.67	–	–
	Trolox	97.50 ± 6.65	21.10 ± 2.94	–
	Ascorbic acid	–	–	16 038.0

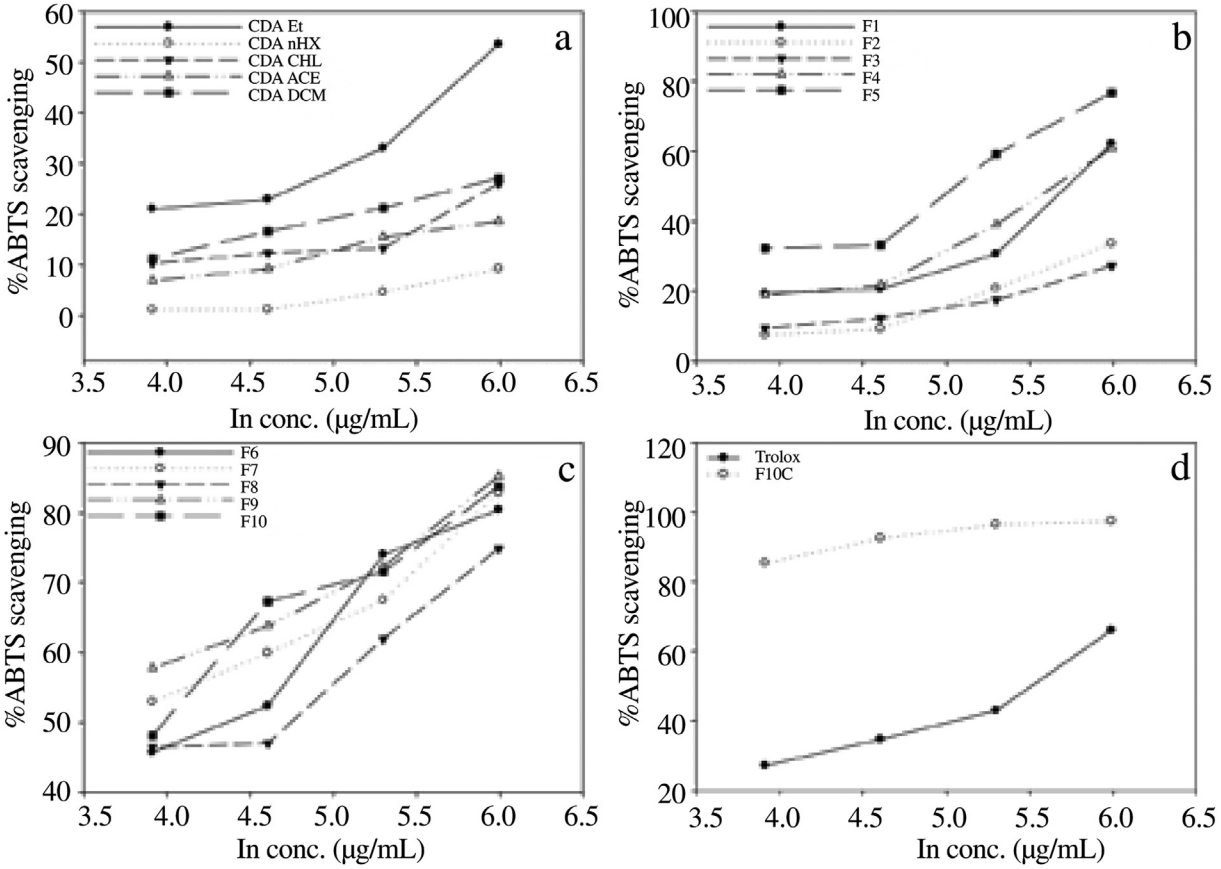

Figure 4. The percentage ABTS scavenging activity of a) various solvent extracts, b) fractions F1−F5, c) fractions F6−F10 and d) trolox and isolated compound.

ABTS radical-scavengers. The presence of flavonoids was indicated in the crude ethanolic extract by two dimensional-paper chromatography (2D-PC) after development with 15% acetic acid in both dimensions and the visualization of the spots under UV light (365 nm).

Phenolic extract (CDA MW) used for fractionation in this study has an IC_{50} value of 1123.9 μg/mL against the ABTS radical. Antioxidant activity of all the solvent extracts was lower as compared to the fractions F1−F10. All the ten fractions exhibited varying degrees of scavenging capacity (Figure 4b and c). At all concentrations (50–400 μg/mL) six fractions F5−F10 showed stronger ABTS radicals scavenging activity than fractions F1−F4. Among these six fractions, the activity of the fractions towards the ABTS radical increased in the order F5 < F8 < F6 < F10 < F7 < F9 (Figure 5). The calculated IC_{50} values for all the fractions against ABTS radicals are given in Table 2. The antioxidant activity of fraction F9 (IC_{50} 31.1 μg/mL) (Figure 5) was approaching that of standard trolox (IC_{50} 21.1 μg/mL).

At 50 μg/mL concentration, the scavenging effect of F10C (5,7,4′-trihydroxy-3′-methoxy flavone) was considerably higher (85.4%) than that of trolox (26.7%) (Figure 4d), indicating its ability to act as a scavenger at low concentrations. F10C (5,7,4′-trihydroxy-3′-methoxy flavone) with lowest IC_{50} value of 0.08 μg/mL (Figure 5 and Table 2) was far more effective than the trolox (IC_{50} 21.1 μg/mL) and all the fractions which showed IC_{50} ranging from 31.1 to 2158 μg/mL. Interestingly F10C also showed significantly stronger ABTS scavenging potency (IC_{50} 0.08 μg/mL) than DPPH scavenging (IC_{50} 43.8 μg/mL).

3.4. Ferric reducing antioxidant power assay

Antioxidant potential of different solvent extracts were estimated by the FRAP method in this study. A dose–response relationship was observed using the FRAP assay; the absorbance increased as the concentration increased for each individual extract (Figure 6a). At 50 μg/mL, the ethanol extract of *C. didymus* (aerial parts) showed the highest reducing capacity (optical density = 0.304) but remained lower than the standard

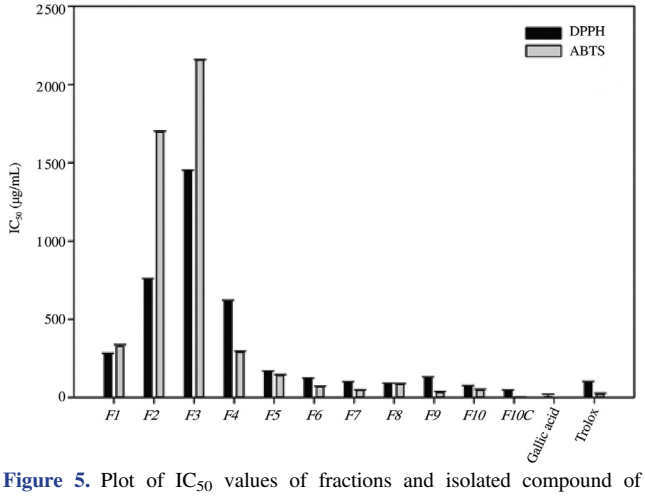

Figure 5. Plot of IC_{50} values of fractions and isolated compound of ethanolic extract of *C. didymus* and standards against DPPH and ABTS radicals.

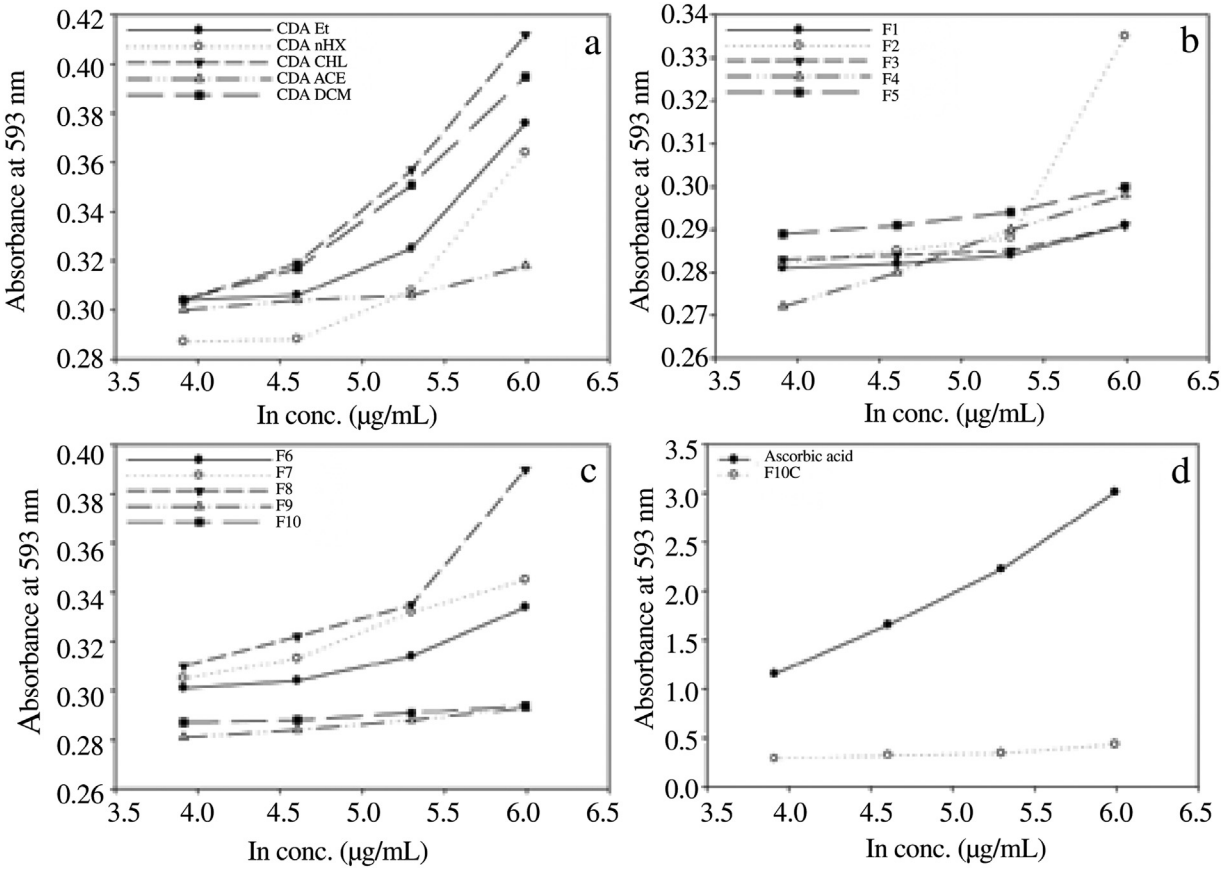

Figure 6. Reducing power activity of a) various solvent extracts, b) fractions F1−F5, c) fractions F6−F10 and d) ascorbic acid and isolated compound.

used which was ascorbic acid (optical density = 1.151). The ethanolic extract was the most potent at all concentrations in reducing the Fe (III)-TPTZ (Figure 6a). Low to medium dose (50–100 µg/mL) of chloroform and acetone extracts (Figure 6a) were more effective than DCM and *n*-hexane extracts in TPTZ scavenging. The Fe (III)-TPTZ reduction by all the fractions were also dose dependent as illustrated in Figure 6b and c. It was found that low to medium doses of four fractions F5−F8 showed stronger TPTZ reduction than all other fractions.

The FRAP values (µM Fe (II) equivalent) of these extracts and fractions from *C. didymus* were determined by constructing a standard curve with $FeSO_4$ taking into consideration the relationship between absorbance and concentration. The calibration curve generated from detection of sample containing known amounts of the standard $FeSO_4$ was linear (Figure 7) with $y = 0.000\ 06x + 0.1887$; $R^2 = 0.989$.

The FRAP values [µM Fe (II) equivalent] were calculated for each extract and fractions using their absorbance values (as y)

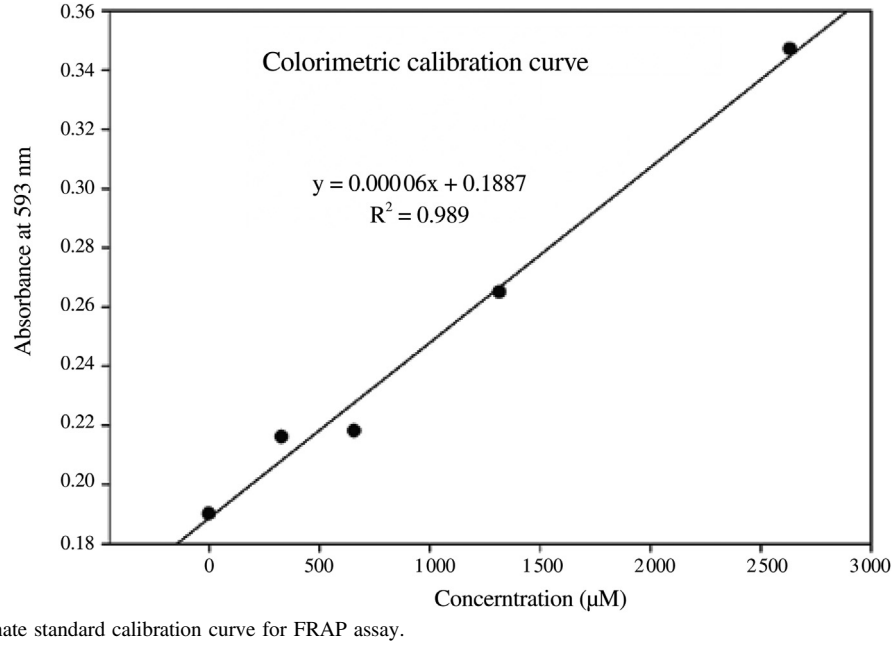

Figure 7. Ferrous sulphate standard calibration curve for FRAP assay.

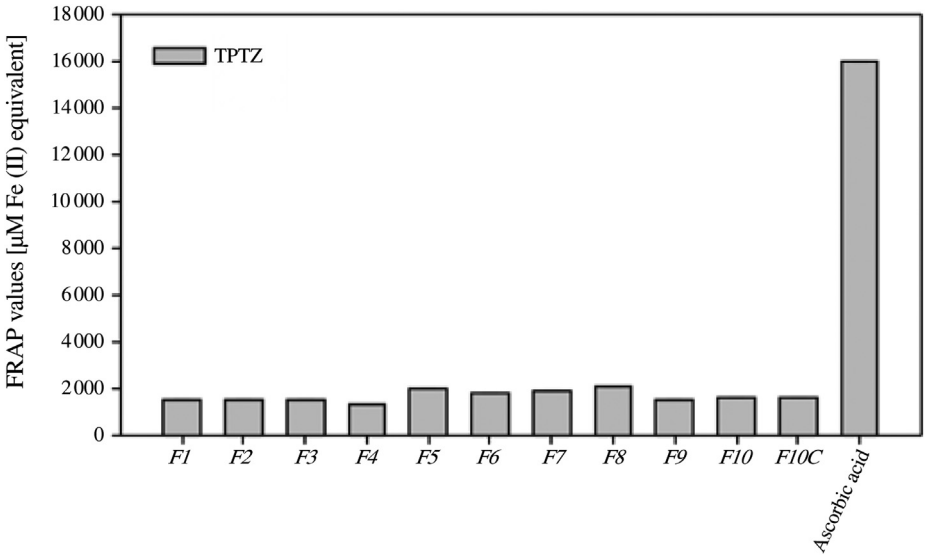

Figure 8. Plot of FRAP values [µM Fe (II) equivalent] of fractions and isolated compound of ethanol extract of *C. didymus* and standard.

and the equation generated by the $FeSO_4$ standard curve. The ferric reducing ability of the various solvent extracts revealed that each showed high FRAP value (1638.3–1921.7 µM Fe (II) equivalent). Among all the extracts, the ethanol, chloroform and DCM extracts showed the highest activity [1921.7 µM Fe (II) equivalent] while *n*-hexane extract [1638.3 µM Fe (II) equivalent] showed the lowest FRAP value (Table 1). Results of the ferric reducing antioxidant power assay shown that all extracts presented closer values, probably associated to synergistic or antagonistic effect of antioxidant compounds present in the samples. Ascorbic acid was used as a standard showing very high TPTZ scavenging activity with 16 038.0 µM Fe (II) equivalent at 50 µg/mL concentration (Table 2).

Four fractions F5−F8 showed stronger TPTZ reduction than other fractions. It was observed that among the fractions F5−F8, the fractions F8 and F5 shows high absorbance at low dose than fractions F6 and F7 (Figure 6b and c). So fractions F8 and F5 that gave a absorbance of 0.310, and 0.309 at 593 nm has a value of 2021 and 2005 µM Fe (II) equivalent, respectively. The FRAP values for all the fractions are given in Table 2. On the whole, the activity of the fractions towards TPTZ reduction increased in the order F6 < F7 < F5 < F8 (Figure 8) in the later collected fractions. The trend indicated the presence of strong electron donating antioxidants in these fractions which reduced ferric ions into ferrous ions under the reaction conditions. The isolated flavone (F10C) has a FRAP value of 1621.7 µM Fe (II) equivalent (Table 2).

4. Discussion

Free radicals are considered to be important causative factors in the development of chronic diseases. Plant polyphenols are extremely important components of the human diet due to their reported antioxidant activity and capacity to alleviate oxidative stress-induced tissue damage which is associated with a number of chronic diseases [17]. Medicinal plants, being the potential source of natural antioxidants have been extensively studied, because some synthetic antioxidants are harmful to human health. *In vitro* antioxidant activity of *C. didymus* extracts prepared with solvents of different polarity, thus having different phenolic composition was determined and compared. It was found that yield percent and total phenolic content of the extracts obtained from *C. didymus* tended to increase with the increasing polarity of the solvents used as extractants. The total phenolic content of extracts was in the order of CDA Et > CDA ACE > CDA MW > CDA nHX > CDA CHL > CDA DCM. As most phenolic compounds are polar permitting so they have been efficiently extracted in high yield, in higher polarity solvent like ethanol. It was observed that, among the various extracts studied, variation in total phenolic content was large, up to 15 fold, highest in ethanol extract and minimum in dichloromethane extract.

It was found that the percentage of DPPH radical scavenging activity of all the extracts was dose dependent. The ordering of extracts in terms of antioxidant activity from highest to lowest was CDA Et > CDA MW > CDA DCM > CDA CHL > CDA ACE > CDA nHX in DPPH assay. The ethanol extract was the most potent antioxidant suggested that the antioxidant activity of *C. didymus* aerial parts is mainly due to more polar constituents, similar to the results previously reported by Do *et al.* [18]. Among the various fractions, the DPPH scavenging effect of fractions F7, F8, and F10, at 100 µg/mL concentration were considerably higher than the trolox, indicating their ability to act as scavengers at low concentrations. Our previous study on LCMS analysis of the aerial parts of *C. didymus* revealed that flavonoids are present in these fractions [10]. In addition, the isolated compound F10C (5,7,4′-trihydroxy-3′-methoxy flavone) was found to be far more effective than the standard (trolox). Thereby providing an ample advocacy of the use of the plant as an alternative source of natural antioxidants.

The same results were obtained in the investigation of ABTS scavenging activity, ethanolic extract possesses significantly stronger ABTS scavenging potency than all the solvent extracts. The results of our investigation show that at all concentrations (50–400 µg/mL) six fractions F5−F10 showed stronger ABTS radicals scavenging activity, the activity of these fractions towards the ABTS radical increased in the order F5 < F8 < F6 < F10 < F7 < F9, which suggested that the elution process using methanol-water via SEC has a strong concentrating effect for radical-scavenging components in these later collected fractions (F6−F10). It was found at 50 µg/mL concentration, the scavenging effect of F10C was considerably

higher than the trolox, indicating its ability to act as a scavenger at low concentrations. Compound F10C isolated from *C. didymus* has previously been identified as 5,7,4′-trihydroxy-3′-methoxy flavone by LC-MS and NMR spectroscopy [10]. The higher activity of isolated compound F10C (5,7,4′-trihydroxy-3′-methoxy flavone) with lowest IC_{50} value (0.08 μg/mL) indicates that flavonoids are responsible for antioxidant activity of *C. didymus* ethanol extract. The isolated flavone (F10C) has higher antioxidant activity which is due to the presence of a free hydroxyl group at 4′-position in the compound. Generally certain structural features in flavonoids are responsible for their high antioxidant activity; the presence of 2,3-unsaturation on the C-ring and the number and substitution of hydroxyl groups on the A and B-rings [19,20], all these features are present in F10C. The antioxidant activity of the phenolic compounds is also attributed to its reducing, hydrogen donors and singlet oxygen quenching properties [21,22].

It is well known that *in vivo* antioxidant activity of phenolic compounds is limited by their absorption and metabolism. Previous study by Wen and Walle revealed that methylated flavonoids are metabolically stable and experienced slower hepatic metabolism compare to the unmethylated flavonoids [23]. The higher activity of the isolated flavonoid F10C (5,7,4′-trihydroxy-3′-methoxy flavone) may thus be due to its methylation, as methylation discharge the effect of metabolizing enzymes, therefore increase the antioxidant activity.

We conclude that the extract and fractions were more effective in ABTS radical scavenging than DPPH, this may be due to complexity, polarity and chemical properties which could lead to varying bioactivity [24]. These results are in accordance with Wang *et al.* which reported that some compounds have high scavenging activity in one assay while concomitantly lower activity in the other assay [25]. Previously Prabhakar *et al.* evaluated free radical scavenging ability of the whole plant aqueous extract of *C. didymus* L., and MeOH−$CHCl_3$ fractions, by DPPH and ABTS assays [11]. They observed that the most non-polar fraction had the highest DPPH and ABTS scavenging activity. As expose above in the present study, the polar ethanolic extract of aerial parts and its polar fractions have the highest antioxidant activity, this variation may be due to extracted parts of plant and climate differences [26].

During the FRAP assay an increase in absorption from all samples, at 593 nm, were observed in comparison to control. Three extracts *i.e.*, CDA Et, CDA CHL and CDA DCM have high ability to reduce Fe^{3+} to Fe^{2+} which can be attributed to reducing agents in these extracts. Two fractions F8 and F5 got significantly higher ferric reducing values than other fractions. Increased absorbance of these two fractions indicated increased reducing power which could serve as a significant indicator of their antioxidant potential. Antioxidant activity of the aerial parts of *C. didymus* by FRAP assay is reported here for the first time, providing an added ground for better understanding of the antioxidant capability of the plant.

Present findings showed a positive relationship between high antioxidant activity and phenolic content for different extracts of this plant, similar to the results previously reported by Gorinstein *et al.* and Wong-Paz *et al.* [27,28]. This study using three different antioxidant methods confirmed that *C. didymus* aerial parts possess remarkable antioxidant activity, which is due to the presence of flavonoids and the high total phenolic content. This indicated that *C. didymus* contained potential antioxidant compounds, therefore, further study of their synergistic effects could provide many chemically interesting and biologically active natural antioxidants.

Conflict of interest statement

We declare that we have no conflict of interest.

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