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Development of a new chromium reducing antioxidant capacity (CHROMAC) assay for plants and fruits

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ABSTRACT

A chromium reducing antioxidant capacity (CHROMAC) assay was presented to measure antioxidant capacity of selected plants and fruits and compared its performance with other commonly used antioxidant capacity methods of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and cupric reducing antioxidant capacity (CUPRAC). The assay is based on the spectrophotometric measurement of colored a chelate complex of Cr(III) and diphenylcarbazone formed by the reaction of Cr(VI) and 1,5-diphenylcarbazid in acidic medium. Phenolic compounds react with excessive amounts of Cr(VI) at low pH values, causing reduction of Cr(VI) to Cr(III) and conversion of phenols to oxidized products. The assay comprises of the antioxidant with a chromium(VI) solution, a 1,5-diphenylcarbazid in acidic medium and subsequent measurement of the developed absorbance at 540 nm after 50 min. The color development is stable for phenolic compounds in plant and fruit. The selectivity of the assay for phenolic compounds was improved by adjusting pH to 2.8 and reduction potential between 0.2 and 0.9 V. The developed assay was successfully applied to the measurement of antioxidant capacity of three plants and one fruit (*Prunus divaricata* Ledeb.subsp. *divaricata*) samples and comparable results were obtained by ABTS and CUPRAC assays.

1. Introduction

Fruits and plants contain a wide range of antioxidant components in plant tissues which are responsible for their antioxidant capacity. These antioxidants include vitamins, carotenoids, phenolic acids and flavonoids which could play an important antioxidant role since they behave as free radical scavengers, singlet and triplet oxygen quenchers, enzyme inhibitors and metal-chelators [1]. Phenolics are reported to inhibit low density lipoprotein (LDL) oxidation and have antioxidant, anticancer, and antiinflammatory activity [2]. Therefore, plants have shown a remarkably high scavenging activity towards generated radicals.

Several methods have been reported for determination of antioxidant capacity in plants and foods. Antioxidant capacity assays can be divided into two mechanisms as single electron transfer (ET) and hydrogen atom transfer (HAT). 2,2-azino-di-(3-ethylbenzothialozine-sulphonic acid) (ABTS) [3, 4], 2,2-diphenyl-1-picrylhydrazyl (DPPH) [5–7], cupric ion reducing antioxidant capacity (CUPRAC) [8] and ferric reducing antioxidant power (FRAP) [9,10] are classified as electron transfer assays; oxygen radical absorbance capacity (ORAC) and total radical absorption potentials (TRAP) are classified as hydrogen atom transfer assays [11]. ET based assays is based on the measurement of the capacity of an antioxidant in the reduction of an oxidant, which generally changes color when reduced. ET reactions are relatively slow and can require a long time to reach completion, so antioxidant capacity calculations are based on percent decrease in product rather than kinetics. On the other hand, HAT based methods measure the classical ability of an antioxidant to quench free radicals by hydrogen donation. HAT reactions are quite rapid and are typically completed in seconds to minutes [12]. Antioxidant capacity methods differ in terms of their assay principle and experimental conditions. Because of multiple reaction characteristics and mechanisms, a single assay will not acurately reflect all antioxidants in a mixed or complex system [13]. Thus, each method provides an estimate of antioxidant activity that is dependent on the type of assay selected and experimental conditions. Therefore, these differences in total antioxidant capacity indicate the need for further standardization of total antioxidant capacity determination methods and the use of different methods help to identify variations in the response of the compounds extracted from the fruit or plant samples.

The CHROMAC is a novel assay as a spectrophotometric total antioxidant capacity assay based on the reaction of excessive Cr(VI) with phenolic compounds and formation of coloured complex with remaining Cr(VI) and diphenylcarbazide. Potassium dichromate, $K_2Cr_2O_7$, is a common inorganic chemical reagent and will be used as source of Cr(VI), most commonly used as an oxidizing agent in various laboratory and industrial applications.

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Aqueous Cr(VI) exists as five species including H_2CrO_4, $HCrO_4^-$, CrO_4^{2-} and $Cr_2O_7^{2-}$. Excess of Cr(VI) indicate that a monomeric species (H_2CrO_4, $HCrO_4^-$ or CrO_4^{2-}) is the primary reactant with phenols. Hydroxylated organic compounds such as phenols are highly reactive with Cr(IV) in aqueous systems. The reaction was first-order with respect to concentrations of both $HCrO_4^-$ and the phenol reductant, and the rate increased with decreasing solution pH [14,15]. The color reaction between chromium(VI) and 1,5-diphenylcarbazide has long been used for the colorimetric determination of chromium, because the assay used to do so can be quite rapid, simple and selective, with the pH kept between 1 and 2 [16]. The Cr(VI) and diphenylcarbazide react in acidic solution to produce an intense red–violet product with an absorption maximum at 540 nm.

The objective of this study was to develop a simple, inexpensive, sensitive and selective indirect spectrophotometric CHROMAC assay for the determination of total antioxidant capacity of different types of food antioxidants. Determination of total antioxidant capacity requires stable reactants to measure the reactions long enough to reach equilibrium. The developed CHROMAC assay has some advantages over the other commanly used antioxidant capacity measurement assays with regard to interference effecets of protein, citric acid and glucose, and time required for measurement.

2. Experimental

2.1. Chemicals and reagents

Gallic acid, (+)-catechin hydrate, quercetin hydrate, rosmarinic acid, rutin, chlorogenic acid, vanillic acid, 2-hydroxycinnamic acid, trans-cinnamic acid, malvidin chloride, 1,5-diphenylcarbazide, neocuproine (2,9-dimethyl-1,10-phenanthroline) and potassium dichromate were obtained from Sigma-Aldrich (St. Louis,Mo., U.S.A.). Trolox and butylated hydroxytoluene (BHT) were obtained from Aldrich (Aldrich Chemicals Co., Steinheim, Germany). Caffeic acid, ferulic acid, *p*-coumaric acid, *p*-hydroxybenzoic acid, protocatechuic acid, sodium dihydrogen phosphate, phosphoric acid, ascorbic acid, ethanol and methanol were purchased from Merck (Darmstadt, Germany). Ellagic acid and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Fluka (Buchs, Switzerland). All standard polyphenolic solutions were prepared in methanol (Merck, Darmstadt, Germany). Ascorbic acid was prepared in water and BHT was prepared in ethanol (Merck, Darmstadt, Germany).

2.2. Plant materials

Laurus nobilis L. (bay laurel) leaves, *Erica vulgaris* L. (heater), *Teucrium polium* L. (felty germander) were obtained from a local market in Bursa—Turkey. *Prunus divaricata* Ledeb.subsp. *divaricata* (red fruit) was collected from region of Bursa—Turkey. It was hand-harvested at mature stage during the growing season. The obtained samples were stored at 4 °C.

2.3. Instrument

Spectrophotometric measurements were performed on a UV/vis spectrometer (Varian Cary-50 UV/vis, in Melbourne, Australia) equipped with 10 mm quartz cuvettes.

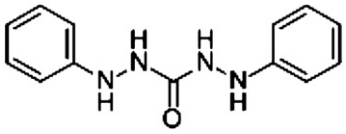
2.4. The scheme of the CHROMAC reactions

The dichromate used as oxidizing reagent of the CHROMAC assay reacts with *n*-electron-reductant antioxidants in the following manner:

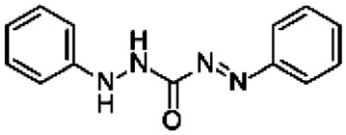
$Cr_2O_7^{2-} + H^+ + n$-electron reductant (phenolics) $\rightarrow Cr^{+3} +$ *n*-electron-oxidized product excessive

$$Cr_2O_7^{2-} + 3H_4L + 6H^+ \rightarrow [Cr^{III}(HL)_2]^+ + Cr^{3+} + H_2L + 7H_2O$$

H_4L Diphenylcarbazide



H_2L Diphenylcarbazone



In the CHROMAC assay; phenolic compounds are oxidized to a phenoxy radical with excessive amounts of Cr(VI) in acidic medium, and Cr(VI) is transformed into Cr(III). The remaining Cr(VI) reacts with 1,5-diphenylcarbazide at pH 2.8 to produce a chelate complex of Cr(III) and diphenylcarbazone at 540 nm [17]. The absorbance of phenolic compounds is calculated in the following way:

$$A_{Sample} = A_{Blank} - A_{Remaining}$$

where A_{Blank} is the absorbance of the control reaction containing all reagents with excessive amounts of Cr(VI) except the phenolic compounds. A_{sample} is the absorbance of phenolic compounds.

2.5. Extraction of phenolic compounds

Plant samples (2 g) were blended with methanol/water (70:30, v/v) at room temperature in the dark for 4 h under a magnetic stirrer. The samples were treated with nitrogen gas before extraction to prevent oxidation of phenolic compounds. The samples (total volume 50 mL) were separated from the solid matrix by filtration through sheets of qualitative filter paper (75 g m^{-2}, 0.2 mm thickness). The extracts were used for determination of total antioxidant capacity by CHROMAC, CUPRAC and ABTS methods.

Fresh fruit samples (7.5 g) were extracted with different solvents including water, methanol, ethanol, methanol/water (50:50, v/v) and ethanol/water (50:50, v/v) at room temperature in dark for 1 h under ultrasonic cleaning bath (United) at 40 kHz. The samples were treated with nitrogen gas before extraction. The samples (total volume 25 mL) were separated from the solid matrix by filtration through sheets of qualitative filter paper. The extracts were used for determination of total antioxidant capacity by CHROMAC, ABTS and CUPRAC methods.

2.6. CHROMAC antioxidant capacity assay

The total antioxidant capacity of plants and fruits were determined by CHROMAC assay. To a test tube, 2 mL phosphate buffer solution (pH 2.8), 0.25 mL $K_2Cr_2O_7$ solution (100 mg L^{-1}), x mL of extract was added. $K_2Cr_2O_7$ is reacted with the antioxidant sample, after incubation for 1 min it was mixed with 0.5 mL 1,5-diphenylcarbazide (3.4×10^{-4} mol L^{-1}), and (2.25–x) mL

water was added to the initial mixture so as to make the final volume of 5 mL. After 50 min, the absorbance of the solution was recorded at 540 nm against the reagent blank. Standard curve was prepared using different concentrations of trolox. The results were expressed as mg TE per g of sample.

2.7. ABTS antioxidant capacity assay

The total antioxidant capacity of plants and fruits were determined with ABTS method, as described in our previous work [18]. $ABTS^+$ was produced by reacting 20 mM ABTS solution with 2.45 mM potassium persulfate solution and allowing the mixture to stand in the dark at room temperature for 12–16 h before use. The procedure for plants was performed by adding x mL extract, (4–x) mL of ethanol and 1 mL of the $ABTS^{\cdot+}$ radical cation solution which was diluted with ethanol at a ratio of 1:10. The absorbance was recorded at 734 nm against the blank after 6 min. Standard curve was prepared using different concentrations of trolox. The results were expressed as mg TE per g of sample.

2.8. CUPRAC antioxidant capacity assay

The cupric ion reducing antioxidant capacity of plants and fruits were determined as described in our previous work [18]. To a test tube, 1 mL each of $CuCl_2$ solution (1.0×10^{-2} mol/L), neocuproine alcoholic solution (7.5×10^{-3} mol/L), and NH_4Ac (1 mol/L, pH 7.0) buffer solution and x mL extract and water were added to the initial mixture so as to make the final volume 4.1 mL. After 30 min, the absorbance was recorded at 450 nm against the reagent blank. Standard curve was prepared using different concentration of Trolox. The results were expressed as mg TE per g of sample.

3. Results and discussion

3.1. Dependence of pH on chromium(VI) oxidazing power

Reaction mechanism between Cr(VI) and antioxidant compound depends strongly on the acidity of the solution. Most antioxidants have a redox potential generally between 0.2 and 0.8 V [19]. Cr(VI) in acidic medium demonstrates a very high positive redox potential ($E^0=1.33$ V) [20] and therefore cannot be selective for antioxidant compounds. The redox potential of Cr(VI) can be reduced by adjusting pH to 2.8 to increase the selectivity only for antioxidants. Cr(VI) forms several species, the relative proportions of which depend on pH and Cr(VI) concentration. Oxidation of antioxidant compounds by Cr(VI) produces reduction products of Cr(III), Cr(IV) and Cr(V). The species of Cr(IV) and Cr(V) are not stable below pH 3 [20]. Cr(III) does not react with antioxidants and 1,5-diphenylcarbazide at this pH. However, Cr(III) reacts with antioxidants between pH 3 and 4 and gives maximum absorbance at 550 nm with the absorbance of a chelate complex of Cr(III) and diphenylcarbazone [21]. Therefore, 2.8 was chosen as appropriate pH for solution to increase the selectivity of the proposed assay.

3.2. Effect of reaction time

The CHROMAC assay is typically run by the following procedure: Excessive amount of $K_2Cr_2O_7$ solution (0.25 mL, 100 mg L^{-1}) in 2 mL buffer solution (pH 2.8) is mixed with sample. After 1 min incubation, the remaining Cr(VI) is reacted with 1,5-diphenylcarbazide (0.5 mL, 3.4×10^{-4} mol L^{-1}) to produce a chelate complex of Cr(III) and diphenylcarbazone. The reaction progress absorbance of the mixture is monitored at 540 nm for 50 min until the absorbance is stable.

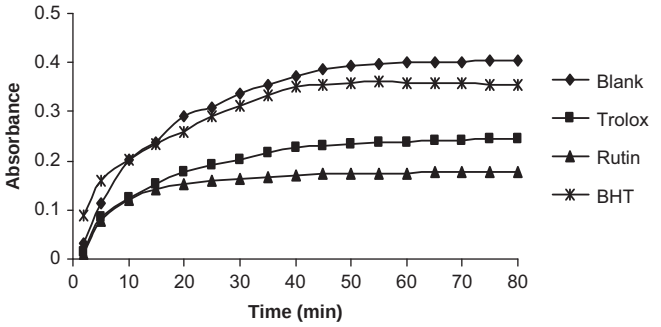


Fig. 1. The effect of the duration of interaction of Cr(VI), specific antioxidants and complexing agent on the absorbance of the tetraaqua(1,5-diphenylcarbazone)-chromium(II) complex at 540 nm for blank, trolox, rutin and BHT.

Fig. 1 illustrates the effects of the duration of interaction of Cr(VI), specific antioxidants and complexing agent on the absorbance of the chelate complex of Cr(III) and diphenylcarbazone at 540 nm for Trolox, the standard reference compound, compared with rutin, BHT (lipophilic antioxidant) and blank. The results demonstrate that the oxidation and complexation reactions are completed in 50 min.

3.3. Concentration ranges of selected antioxidant compounds

The concentration ranges, regression equations and Trolox equivalent antioxidant capacity (TEAC) of selected antioxidants with respect to the proposed assay are given in Table 1. The equations for the calibration line of antioxidants with five points are in the concentration ranges of 6.0×10^{-5} and $4.5\times10^{-}4$ mol L^{-1} gave linear relationship of absorbance versus concentration, where R^2 was above 0.9901. Vanillic acid yielded the highest molar absorptivity as 721 L mol^{-1} cm^{-1}. The results show that the method is sensitive for phenolic acids, flavonoids and synthetic antioxidants.

Antioxidant capacities of the selected phenolic acids, flavonoids, malvidin, ascorbic acid and BHT are shown in Table 1. All flavonoids demonstrated higher TEAC coefficients than phenolic acids as obtained in literature [22]. The TEAC coefficients obtained are close to those obtained by CUPRAC and ABTS assays except for gallic acid, ellagic acid, quercetin and (+)-catechin [10, 22]. The developed method gave lower TEAC coefficients than CUPRAC and ABTS methods for these compounds. It can be concluded from the experimental results that further oxidation of gallic acid and quercetin with Cr(VI) was under control at acidic medium. The TEAC coefficient in the CHROMAC assay was the highest among flavonoids for quercetin. Rutin, having an *o*-rutinase substituent instead of –OH in the 3-position, showed lower antioxidant capacity. The relatively high TEAC coefficient of quercetin could be attributed to the fact that rutin, a glycoside of quercetin, was hydrolysed in acidic condition to the aglycone. The TEAC coefficients for hydroxycinnamic acids with respect to the CHROMAC and ABTS assays (ABTS assay in parantheses) are as follows: *p*-cumaric acid 2.28 (2.22), ferulic acid 2.20 (2.20), chlorogenic acid 2.15 (1.20), ellagic acid 1.90 (1.21), gallic acid 1.70 (3.00) and caffeic acid 1.55 (1.40). The TEAC order for these phenolic compounds are similar with those of widely used ABTS assay but it is opposite with CUPRAC assay [10,23]. *P*-cumaric acid and ferulic acid exhibit higher TEAC coefficients that contains one phenolic –OH group than two –OH containing chlorogenic acid and caffeic acid. This may be explained by the steric hindrance of the more –OH groups together with the overall extent of conjugation in the molecule such as gallic acid, ellagic acid and rosmarinic acid. Small molecules may have a better chance the radical with subsequently higher TEAC coefficients.

Table 1
Concentration ranges of various antioxidant compounds with the CHROMAC assay.

Antioxidant compounds	Concentration range (mol L^{-1})	Regression equation	Linearity (R^2)	TEAC coefficients
Gallic acid	1.2×10^{-4}–4.5×10^{-4}	$y=253.18x+0.0596$	0.9989	1.70
Trolox	3.0×10^{-4}–7.0×10^{-4}	$y=566.38x-0.1369$	0.9994	1.00
Caffeic acid	3.0×10^{-4}–4.5×10^{-4}	$y=593.86x-0.1125$	0.9997	1.55
Ferulic acid	2.5×10^{-4}–4.0×10^{-4}	$y=361.8x-0.0681$	0.9901	2.20
Ellagic acid	3.0×10^{-4}–4.5×10^{-4}	$y=419.57x-0.0686$	0.9908	1.90
Chlorogenic acid	2.5×10^{-4}–4.5×10^{-4}	$y=412x-0.0999$	0.9933	2.15
Rosmarinic acid	2.0×10^{-4}–4.5×10^{-4}	$y=220.71x+0.0779$	0.9921	1.71
Protocatechuic acid	6.0×10^{-5}–1.0×10^{-4}	$y=337.5x+0.096$	0.9995	0.97
Vanillic acid	3.0×10^{-4}–4.0×10^{-4}	$y=721x-0.1529$	0.9976	1.44
2-Hydroxycinnamic acid	3.0×10^{-4}–4.5×10^{-4}	$y=442.86x-0.0806$	0.9999	1.88
p-Hydroxybenzoic acid	3.0×10^{-4}–4.5×10^{-4}	$y=433.4x-0.0937$	0.9911	2.00
p-Coumaric acid	2.0×10^{-4}–4.5×10^{-4}	$y=247.32x+0.0129$	0.9975	2.28
Trans-cinnamic acid	3.0×10^{-4}–4.5×10^{-4}	$y=440x-0.0631$	0.9990	1.77
Rutin	3.0×10^{-4}–4.5×10^{-4}	$y=369x-0.0815$	0.9989	2.26
Quercetin	3.0×10^{-4}–4.5×10^{-4}	$y=312.14x-0.0661$	0.9999	2.53
(+)-catechin	3.5×10^{-4}–4.5×10^{-4}	$y=422x-0.1418$	0.9999	2.38
Malvidin	3.0×10^{-4}–4.5×10^{-4}	$y=486.71x-0.1217$	0.9968	1.95
Ascorbic acid	8.3×10^{-5}–1.3×10^{-4}	$y=23.6x+0.1460$	0.9936	2.72
BHT	1.2×10^{-4}–4.0×10^{-4}	$y=350.5x+0.0381$	0.9982	1.40

3.4. Application of the method to real samples

The developed indirect spectrophotometric assay of antioxidant capacity is based on the redox reaction between phenolic compounds and excessive amount of Cr(VI) at pH 2.8 and measurement of absorbance after raction of remaining Cr(VI) with 1,5-diphenylcarbazide. The standard redox potential of Cr(VI) is too high that the compounds other than plant and fruit antioxidants of interest, like glucose or citric acid, may also be oxidized within sufficient time causing positive error. Therefore, solution acidity should be properly adjusted such that oxidation power of the Cr(VI) is kept in the range of antioxidants oxidation potentials to increase selectivity and eliminate the interference effects of chromium species produced during antioxidant oxidation. On the other hand, the absorbance wavelength of the reaction between Cr(III) and phenolic compounds should not shift with a chelate complex of Cr(III) and diphenylcarbazone. The reaction of phenolic compounds with Cr(III) in low acidic aquous (pH 3–4) solutions give a maximun absorbance at about 550 nm [21]. So we eliminated the reaction of Cr(III) with phenolic compounds by reducing the pH to 2.8 and measured the absorbance at 540 nm. Simple sugars, citric acid and amino acids, which are not true antioxidants, are not oxidized in the CHROMAC assay. We verified these interfering substances on the CHROMAC assay. To test the interfering substances of non antioxidants, 0.1 and 0.15 mM glycine, citric acid and glucose were added separately to 0.3 mM Trolox solutions and antioxidant capacity was measured following the above reported procedure (Table 2). From the experimental data it appears that the observed antioxidant capacity with the CHROMAC assay remained at about 0.70, showing that the Cr(VI) at the optimized concentration and acidity did not oxidized glycine, citric acid and glucose in the 0.2–0.6 V standard potential range of interest. It should be indicated that all Fe(III)-based assays including FRAP are affected by citric acid due to their redox potential [24]. On the other hand, some antioxidant assays such as CUPRAC are not interferred by citric acid [25]. We can conclud that the proposed method is considerably selective toward true antioxidants at pH 2.8.

Anthocyanins, one of the most important phenolic compounds in fruit and other plant tissues also have maximum absorbance at 540 nm [26]. Anthocyanins have different chemical forms which depend on the pH of the solution, the flavylium cation (red colour) is the predominant species at pH 1 and contributes to purple and red colours. The quinoidal blue species of anthocyanins are also predominant between pH 2 and 4 [27]. The absorbance spectra of

Table 2
The antioxidant capacity values obtained from CHROMAC assay containing 0.1 and 0.15 mM glycine, citric acid and glucose. (Data are expressed as mM Trolox).

Substances	Antioxidant capacity
Trolox	0.70
Trolox+0.1 mM glycine	0.70
Trolox+0.15 mM glycine	0.70
Trolox+0.1 mM citric acid	0.70
Trolox+0.15 mM citric acid	0.69
Trolox+0.1 mM glucose	0.69
Trolox+0.15 mM glucose	0.69

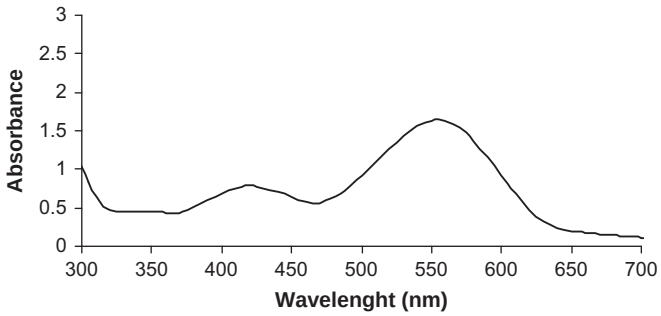


Fig. 2. The absorbance spectrum of pelargonidin at pH 2.8. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pelargonidin and fruit samples (*P. divaricata* Ledeb.subsp. *divaricata* (red fruit)) at pH 2.8 were shown in Figs. 2 and 3, respectively. The maximum absorbance of pelargonidin is 555 nm at pH 2.8 in Fig. 2. *P. divaricata* Ledeb.subsp. *divaricata* (red fruit) samples have no absorbance at 540 nm as shown in Fig. 3. It was understood that the anthocyanins have no interference effect on CHROMAC assay at pH 2.8.

CHROMAC assay was applied to three different plants (Table 3) and one fruit (Table 4) materials. The results represent the antioxidant capacity values after subtraction of methanol and ethanol contributions, which were used during the extraction of phenolic compounds in plants and fruit. The Trolox-equivalent antioxidant capacities of three herbal samples (*L. nobilis* L., *E. vulgaris* L. and *T. polium* L.) and one wild plum (*P. divaricata* Ledeb.subsp.

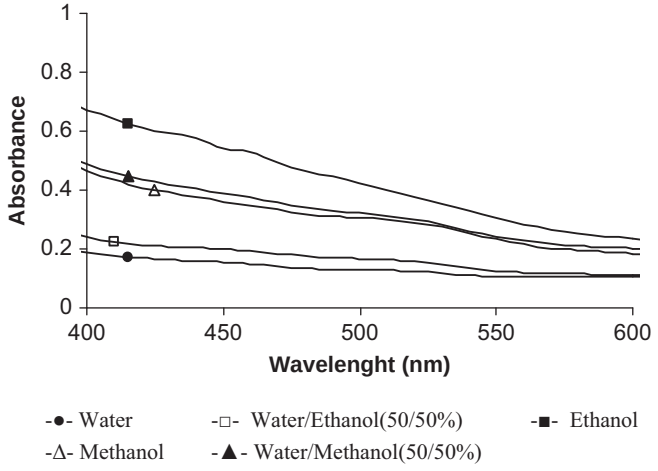


Fig. 3. The absorbance spectrum of *P. divaricata* Ledeb.subsp. *divaricata* extracts (red fruit) at pH 2.8. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 3
Antioxidant capacity of three plants determined by CHROMAC, CUPRAC and ABTS assays.

Plant samples	Antioxidant capacity (mg TE/g dried plant)		
	CHROMAC	CUPRAC	ABTS
Laurus nobilis L.	8.68 ± 0.06	8.45 ± 0.03	9.69 ± 0.13
Erica vulgaris L.	5.13 ± 0.16	5.21 ± 0.02	5.19 ± 0.03
Teucrium polium L.	10.44 ± 0.01	9.97 ± 0.01	12.64 ± 0.29

Table 4
Antioxidant capacity of *Prunus divaricata* Ledeb.subsp. *divaricata* determined by CHROMAC, CUPRAC and ABTS assays.

Prunus divaricata L.	Antioxidant capacity (mg TE/g dried plant)		
	CHROMAC	CUPRAC	ABTS
Water	2.79 ± 0.06	2.77 ± 0.01	1.98 ± 0.11
Methanol	5.16 ± 0.16	4.86 ± 0.02	4.38 ± 0.30
Ethanol	5.27 ± 0.01	4.89 ± 0.03	4.16 ± 0.29
Water/methanol (50/50%)	4.63 ± 0.02	4.56 ± 0.04	3.78 ± 0.24
Water/ethanol (50/50%)	2.59 ± 0.01	2.62 ± 0.01	2.60 ± 0.02

divaricata) (red fruit) measured with the proposed assay were compared with those found by CUPRAC and ABTS methods. The antioxidant capacity results of the developed method are comparable with those of CUPRAC and ABTS reference methods indicating that the CHROMAC assay can be applied as electron-transfer based antioxidant capacity measurement method for phenolic compounds in plants and fruits. The differences of the results are due to the diversity of reaction conditions (pH, redox potential, etc.) of these assays. The highest antioxidant capacity values were determined in the plant extracts of *T. polium* L. with all three assays. The choice of extraction solvents is a crucial step for the phytochemical analysis and the assessment of antioxidant capacity of extracts. The use of polar solvents was preferred because *L. nobilis* L. [28] is used as a valuable spice and flavouring agent in culinary and food industry, *E. vulgaris* L. [29] and *T. polium* L. [30–32] are consumed as herbal tea and the hydrophilicity is as desirable property for potential drugs. The results showed that water/methanol extraction system provided higher antioxidant capacity with 8.68 mg TE/g dried plant for *L. nobilis* L., 5.13 mg TE/g dried plant for *E. vulgaris* L. and 10.44 mg TE/g dried plant for *T. polium* L. using CHROMAC assay (Table 3). In the present study, methanol, ethanol and mixture of water/methanol (50/50%, v/v) extracts possessed the strongest antioxidant capacities for *P. divaricata* Ledeb.subsp. *divaricata* (red fruit), showing CHROMAC values of 5.16, 5.27 and 4.63 mg TE/g dried plant, respectively (Table 4). These results indicate that the polar solvents are powerful in recovering antioxidants in these plants. The antioxidant capacity of spices, herbs and fruits has been mainly attributed to phenolic compounds. Santoyo et al. [28] reported that linear regression analysis showed a statistically significant correlation between the antioxidant capacity and the amount of total phenolic compounds of *L. nobilis* L. Ethanolic extract of *E. vulgaris* L. showed high antioxidant capacity which was attributed to kaempferol-3-*O*-β-D-galactoside [29]. The antioxidant capacity measurements have been shown that poliumoside, rutin, apigenin are the main compounds of methanol extract of *T. polium* L. and the antioxidant capacity is attributed to these phenolic compounds [31,32].

There are several ET-based assays for measuring the antioxidant capacity of plants and fruits that contain high quantity of antioxidant compounds. ABTS, DPPH, FRAP and CUPRAC are the most common ET-based assays for determining in vitro antioxidant capacity. The CHROMAC is one of the ET-based assays that the excessive Cr(VI) reacts with *n*-electron reductant antioxidant to form phenoxy radical and the remaining Cr(VI) is reduced to the highly colored chelate complex of $[Cr^{III}(HL)_2]^+$ showing maximum absorption at 540 nm. The ABTS and DPPH measure the free radical scavenging capacity of a sample, while CHROMAC, FRAP and CUPRAC measure the ability of a sample to reduce metal oxidants. The pH values have an important effect on the reducing capacity of antioxidants and oxidation power of oxidant. The reducing capacity may be suppressed due to protonation on antioxidants at acidic conditions, whereas in basic conditions, proton dissociation of antioxidant compounds would enhance the reducing capacity of a sample. Therefore, redox potantials of ABTS and CUPRAC assays may not be enough to oxidize antioxidants at acidic conditions. The CHROMAC assay has distinct advantages over the most comman antioxidant capacity assays such as availability and stability of reagents, simplicity of the method, repeatibility and reproducibility over a wide range of concentration, applicability to hydrophilic phenolic compounds and completion of the redox reaction within reasonable time. The CHROMAC is selective in the presence of Cr(III) stabilizing ligand of 1,5-diphenylcarbazide at acidic conditions with the redox potential of about 0.9 V. Simple sugars, citric acid and amino acids, which are not true antioxidants, are, not oxidized in the CHROMAC assay. Interference effects of anthocyanins which are common compounds in colored fruits are eliminated at optimized pH and measurement at 540 nm. However, carotenoids are reported to interfere in the 515 nm absorbance measurement of DPPH [33–35]. Another advantage of CHROMAC was that extracts reacted rapidly with CHROMAC (50 min), whereas the ABTS (2 h) and DPPH reaction took much longer (24 h) [36,37]. The oxidation does not stop at 4 min with FRAP, it slowly progresses even after several hours [12,38]. The FRAP values of some antioxidants cannot be obtained accurately if 4 min reaction time was followed. Many antioxidants that react quickly with peroxyl radicals may be inert to DPPH. The reaction kinetics between CHROMAC and antioxidants are linear between 6.0×10^{-5} and 7.0×10^{-4} concentration. A further disadvantage of DPPH is narrow linear range of absorbance versus concentration and steric inaccessibility of large molecules [39]. The absorbance of DPPH radical at 517 nm after reaction with antioxidants is decreased by light, oxygen, pH and type of solvent [23]. Flavonoid glycosides needed to be hydrolyzed to their corresponding aglycons for fully exhibiting their antioxidant potency. Slow reacting antioxidants needed elevated temperature incubation so as to complete their oxidation with the ABTS, CUPRAC and FRAP

reagents [10,23]. The CHROMAC measures the flavonoids in hydrolyzed conditions at room temperature. The CHROMAC assay proved to correlate well with ABTS and CUPRAC assays in plant and fruit extracts. However, FRAP measures only hydrophilic antioxidants, while DPPH detects only those soluble in organic solvents, especially alcohols [39].

4. Conclusions

This work reports the development of a simple, low-cost, selective, sensitive and diversely applicable spectrophotometric assay for the determination of antioxidant capacity of plants and fruits. The assay is based on the oxidation of phenolic compounds with excessive Cr(VI) at room temperature. The Cr(VI) reducing capacity of the sample is measured under carefully adjusted pH such that only antioxidants would be oxidized and not other chromium species react with phenolic compounds and complexing agent. The proposed assay was applied to measurements of antioxidant capacity of a number of herbal and fruit extracts and the results were compared with those of ABTS and CUPRAC assays. The assay has several advantages such that the selected chromogenic reagent is easily accessible, stable, selective, and capable of responding to many types of food antioxidants such as flavonoids, simple phenolic acids and hydroxycinnamic acids. In conclusion, the developed assay is suitable for routine use in laboratories and should be utilized for rapid analysis of food extracts. Future directions of this work are to investigate the applicability of the assay to different plant and food samples with respect to structural properties of phenolic compounds.

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