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Antioxidant Activities of Phenolic, Proanthocyanidin, and Flavonoid Components in Extracts of *Cassia fistula*

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Cassia fistula L., a semi-wild Indian Labernum, is widely cultivated in Mauritius as an ornamental tree for its beautiful bunches of yellow flowers and also used in traditional medicine for several indications. The total phenolic, proanthocyanidin, and flavonoid contents, and the antioxidant activities, of fresh vegetative and reproductive organs of *Cassia fistula* harvested at different stages of growth were determined using the Trolox equivalent antioxidant capacity (TEAC) and ferric-reducing antioxidant power (FRAP) assays. The antioxidant activities were strongly correlated with total phenols (TEAC $r = 0.989$; FRAP $r = 0.951$) in all organs studied, and with proanthocyanidins (TEAC $r = 0.980$; FRAP $r = 0.899$) in reproductive organs including fruits. The antioxidant activities of reproductive parts were higher than those of the vegetative organs, with the pods having highest total phenolic, proanthocyanidin, and flavonoid contents and antioxidant potentials (TEAC = 992 ± 0.4 μmol/g dry weight; FRAP = 811 ± 23 μmol/g dry weight).

KEYWORDS: ***Cassia fistula*; total phenolics; flavonoids; proanthocyanidins; antioxidant activity; TEAC; FRAP**

INTRODUCTION

There is a great deal of evidence indicating that excessive free radical production and lipid peroxidation are actively involved in the pathogenesis of a wide number of diseases, including atherosclerosis (*1*), cardiac and cerebral ischemia (*2*), neurodegenerative disorders (*3*), carcinogenesis (*4*), diabetes (*5*−*7*), and rheumatic disorders (*8*), and that they play a major role in the aging process (*9*, *10*). Plant-derived antioxidants such as vitamin E, vitamin C, polyphenols including phenolic acids, phenolic diterpenes, flavonoids, catechins, procyanidins, and anthocyanins are becoming increasingly suggested as important dietary factors (*11*−*13*). Supplementation with berry juice (*14*), flavones from skullcap, catechins from green tea, anthocyanins from chokeberry, and condensed tannins from faba beans (*15*) are indeed protective of oxidative stress indices in rats. Furthermore, the growing interest in the substitution of synthetic food antioxidants by natural ones has fostered research on plant sources and the screening of raw materials for identifying new antioxidants. In this regard polyphenols are being increasingly reported to exhibit quality-preserving and antioxidant effects on foods (*16*−*18*) and edible oils (*19*).

Cassia fistula L., a semi-wild Indian Labernum also known as the Golden Shower, is widely cultivated in Mauritius as an ornamental tree for its beautiful bunches of yellow flowers. It is highly reputed for its strong laxative and purgative properties (*20*). In Ayurvedic medicine, it is used against various disorders such as haematemesis, pruritus, leucoderma, and diabetes (*21*, *22*). The antipyretic, analgesic effects (*23*) and antitussive potentials (*24*) of the plant have also been reported, together with their antifungal and antibacterial activities (*25*). The plant extract is also recommended as a pest-control agent (*26*). These effects have been mainly attributed to the presence of alkaloids (*27*), triterpene derivatives (*28*), anthraquinone derivatives (*29*, *30*), and polyphenolics comprising flavonoids (*31*, *32*), catechins, and proanthocyanidins (*31*−*34*).

It is becoming clear that for in vitro and in vivo characterization of antioxidant propensities, no one method can give a comprehensive prediction of antioxidant efficacy. So use of more than one method is recommended, and there should be greater caution in extrapolating the in vitro data (*35*−*37*). The spectrophotometric technique, total antioxidant activity (TAA), or the Trolox equivalent antioxidant activity (TEAC) involves the generation of the long-lived specific radical cation chromophore of 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by controlled chemical oxidation. The $ABTS^{\bullet+}$ radical cation has absorption maxima in the near-infrared region at 645, 734, and 815 nm. The TEAC reflects the ability of hydrogen- or

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Table 1. Dates of Harvest and Corresponding in Vivo Vegetative and Reproductive Plant Organs Collected

developmental stage	date of collection (1998)
vegetative	
young leaves	June 1
old leaves	June 1
twigs	June 1
bark	June 1
reproductive	
flower buds	Nov 17
flowers	Dec 2
pods (fruits)	Dec 27

electron-donating antioxidants to scavenge the $ABTS^{\bullet+}$ radical cation compared with that of Trolox. The antioxidant suppresses the A_{734} to an extent, and on a time scale, dependent on the antioxidant activity (*38*, *39*). The ferric reducing/antioxidant power (FRAP assay) is widely used in the evaluation of the antioxidant components of dietary polyphenols. The FRAP assay depends on the reduction of a ferric tripyridyltriazine [Fe(III)-$TPTZ)_2$] complex to the ferrous tripyridyltriazine [Fe(II)-$TPTZ)_2$] by an antioxidant, usually a nonphysiological condition with low pH of about 3.6.

Our research on natural polyphenol-rich plant extracts (*40*−*42*), is focused on the study of the relationship between the antioxidant capacity and the total phenolic, proanthocyanidin, and flavonoid contents of vegetative and reproductive organs of *Cassia fistula*, harvested at different stages. In this paper, we report the application of the FRAP and TEAC assays to assess the free radical scavenging capacities and the reducing potentials of the antioxidant constituents of *Cassia fistula* extracts.

MATERIALS AND METHODS

Plant Material. Vegetative and reproductive organs of *Cassia fistula* at different stages of growth were collected on the same plant on the University of Mauritius Campus at Réduit, during 1998. They were immediately weighed and kept at −20 °C for extraction. **Table 1** shows the different plant organs used and their dates of collection.

Extraction. Plant material (50 g) was extracted first with acetone/water (70:30 v/v) (2 × 100 mL) and finally with methanol 100% (2 × 100 mL). Filtrates were concentrated in vacuo at 37 °C, and the resulting aqueous extract was washed with dichloromethane (2 × 100 mL) to remove lipids and chlorophylls before being freeze-dried. This freeze-dried extract was divided into two parts. Part 1 and part 2 were dissolved in distilled water and absolute methanol, respectively, at a final 1:5 fresh weight/volume ratio. Part 1, corresponding to 25 g fresh weight, was used for the antioxidant assays, while Part 2, corresponding to 25 g fresh weight, was utilized for phenolic analyses.

Thin-Layer Chromatography. Total vegetative and reproductive plant extracts were examined by one-dimensional thin-layer chromatography on silica gel plates (Merck). Proanthocyanidins were analyzed after migration in toluene−acetone−formic acid (3:3:1, v/v/v) (*43*) and visualized by vanillin−HCl spray reagent. Flavonoids were separated in ethyl acetate−formic acid−water (8:1:1, v/v/v) and revealed by 1% 2-aminoethyldiphenyl borate solution in methanol followed by 5% poly-(ethylene glycol) 4000 in absolute ethanol at 365 nm (*44*).

Determination of Total Phenolic Content. The Folin−Ciocalteu method (*45*) was used for the estimation of the total phenolic content of the different plant organs. A volume of diluted samples (0.25 mL) was added to 3.5 mL of distilled water in screw-capped test tubes followed by addition of 0.5 mL of Folin−Ciocalteu solution. After 3 min, 1 mL of sodium carbonate (20%) was added, and the test tubes were properly shaken before they were incubated in a boiling water bath for 1 min. The tubes were then allowed to cool in darkness. A blue coloration was developed, and the absorbance was read at 685 nm. Results were expressed in mg of gallic acid equivalent/g dry mass plant material.

Determination of Total Proanthocyanidin Content. A modified acid/butanol assay (*46*) was adopted for quantification of the total proanthocyanidin content of the methanolic plant extracts. A 0.25-mL aliquot of extract was added to 3 mL of a 95% solution of *n*-butanol/HCl (95:5 v/v) in stoppered test tubes, followed by addition of 0.1 mL of a solution of $NH_4Fe\ (SO_4)_2 \cdot 12H_2O$ in 2 M HCl. The tubes were incubated for 40 min at 95 °C. A red coloration was developed, and absorbance was read at 550 nm. The proanthocyanidin content was expressed in mg of cyanidin chloride equivalent/g dry weight of plant material.

Determination of Total Flavonoid Content. The $AlCl_3$ method (*44*) was used for determination of the total flavonoid content of the methanolic extracts. Aliquots of 1.5 mL of extracts were added to equal volumes of a solution of 2% $AlCl_3 \cdot 6H_2O$ (2 g in 100 mL methanol). The mixture was vigorously shaken, and absorbance was read at 367.5 nm after 10 min of incubation. Flavonoid contents were expressed in mg quercetin equivalent/g dry weight.

TEAC Assay. The Trolox equivalent antioxidant capacity (TEAC) method is based on the ability of an antioxidant to scavenge the preformed radical cation $ABTS^+$ relative to that of the standard antioxidant Trolox C. The $ABTS/MnO_2$ method (*38*) was used for evaluation of the TEAC of vegetative and reproductive plant organs. The $ABTS^+$ radical was generated by a reaction between ABTS (0.5 mM) and activated MnO_2 (1 mM) in phosphate buffer (0.1 M, pH 7). To 3 mL of the $ABTS^+$ solution 0.5 mL of plant extracts was added and the decay in absorbance at 734 nm was followed for 15 min. In controls distilled water was used. TEAC values were expressed in μmol Trolox/g dry weight for plant extracts in mmol/L for reference standards. All analyses were made in triplicates.

FRAP Assay. The ferric reducing antioxidant power (FRAP) assay measures the antioxidant potential of "antioxidants" to reduce the Fe^{3+}/tripyridyl-s-triazine complex present in stoichiometric excess to the blue ferrous form. The FRAP assay (*47*) was performed as previously described. FRAP reagent was freshly prepared by mixing together 10 mM 2,4,6-tripyridyl-s-triazine (TPTZ) and 20 mM ferric chloride in 0.25 M acetate buffer, pH 3.6. Plant sample (100 μL) was added to 300 μL of water followed by 3 mL of FRAP reagent at 1 min intervals. The absorbance was read at 593 nm after 4 min incubation at ambient temperature against distilled water. A calibration curve of ferrous sulfate (100−1000 μmol/L) was used, and results were expressed in μmol Fe (II)/g dry weight extract for plant total extracts and in mmo1/L for reference standards from three determinations.

Statistical Analysis. Results are expressed as mean value ± standard deviation ($n = 3$). Simple regression analysis was performed to calculate the dose−response relationship of standard solutions used for calibration as well as of the test samples. Linear regression analysis was performed, quoting the correlation coefficient r_{xy}.

RESULTS

The TEAC values of vegetative organs ranged from 93 ± 5.6 to 157 ± 0.7 μmol/g dry weight, whereas those of reproductive organs were generally much higher and varied between 453 ± 1.0 and 992 ± 0.4 μmol/g dry weight (**Table 2**). The greatest activity in the vegetative organs was measured in the bark, whereas flower buds and pods were characterized by relatively elevated TEAC values (893 ± 1.9 and 992 ± 0.4 μmol, respectively) compared to that of flowers (453 ± 1.0 μmol/g dry weight). Data obtained using the FRAP assay confirmed the patterns obtained using TEAC. Both assays showed that in the vegetative organs, the bark had the highest antioxidant potential, followed by the old leaves, the young leaves, and the twigs. In the reproductive organs TEAC and FRAP results showed that the pods were the most antioxidant organs followed by flower buds and flowers (**Table 2**). The values have been compared with the antioxidant index of known antioxidants run under the same conditions (**Table 3**).

Table 2. Polyphenolic Contents (mg/g Dry Weight) and Antioxidant Activities (FRAP and TEAC Values) in Vegetative and Reproductive Organs of the Total Extracts of *Cassia fistula*

plant organ	total phenolics[a]	total flavonoid[b]	total proanthocyanidins[c]	TEAC[d]	FRAP[e]
young leaves	11 ± 0.2	9 ± 0.1	2 ± 0.2	98 ± 4.3	51 ± 3.2
old leaves	12 ± 0.3	6 ± 0.1	3 ± 0.3	102 ± 2.4	64 ± 0.3
twigs	9 ± 0.3	3 ± 0.7	2 ± 0.2	93 ± 5.6	64 ± 0.9
bark	13 ± 0.1	4 ± 0.2	2 ± 0.1	157 ± 0.7	95 ± 23.3
flower buds	44 ± 1.2	8 ± 0.3	20 ± 2.8	893 ± 1.9	380 ± 3.8
flowers	32 ± 2.4	8 ± 0.3	14 ± 2.1	453 ± 1.0	317 ± 0.8
pods	54 ± 4.2	14 ± 1.3	21 ± 1.0	992 ± 0.4	811 ± 23

[a] Expressed as mg gallic acid equivalent/g dry weight. [b] Expressed as mg quercetin equivalent/g dry weight. [c] Units of mg cyanidin chloride equivalent/g dry weight. [d] In units of μmol/g dry weight. [e] In units of μmol Fe(II)/g dry weight.

Total phenols, proanthocyanidins and flavonoids were determined for all vegetative and reproductive organs. **Figures 1** and **2** depict the influence of proanthocyanidins and flavonoids on antioxidant capacity. The correlation coefficients between total phenols and antioxidant capacity were for TEAC, $r = 0.989$; and FRAP, $r = 0.951$. In vegetative organs, the twigs with the lowest phenolic content (9 ± 0.3 mg/g dry weight) had the lowest antioxidant activity (TEAC = 93 ± 5.6 μmol/g dry weight) whereas the bark with the highest phenolic level (13 ± 0.1 mg/g dry weight) exhibited the highest antioxidant capacity (TEAC = 157 ± 0.7 μmol/g dry weight) (**Table 2**). In reproductive organs, flowers with the lowest phenolic content (32 ± 2.4 mg/g dry weight) had the lowest activity (TEAC =

Table 3. Relative Antioxidant Activities of Selected Reference Standards Assayed Using TEAC and FRAP Methods

standard	TEAC (mmol/L)	FRAP (mmol/L)
Trolox C	1	1.6 ± 0.07
kaempferol	0.96 ± 0.02	0.66 ± 0.02
(+) catechin	3.26 ± 0.09	2.3 ± 0.03
(–) epicatechin	3.12 ± 0.15	2.76 ± 0.03
BHT		0.07 ± 0.02
BHA	0.88 ± 0.01	5.8 ± 0.24
ergothioneine	0.71 ± 0.02	0.46 ± 0.06

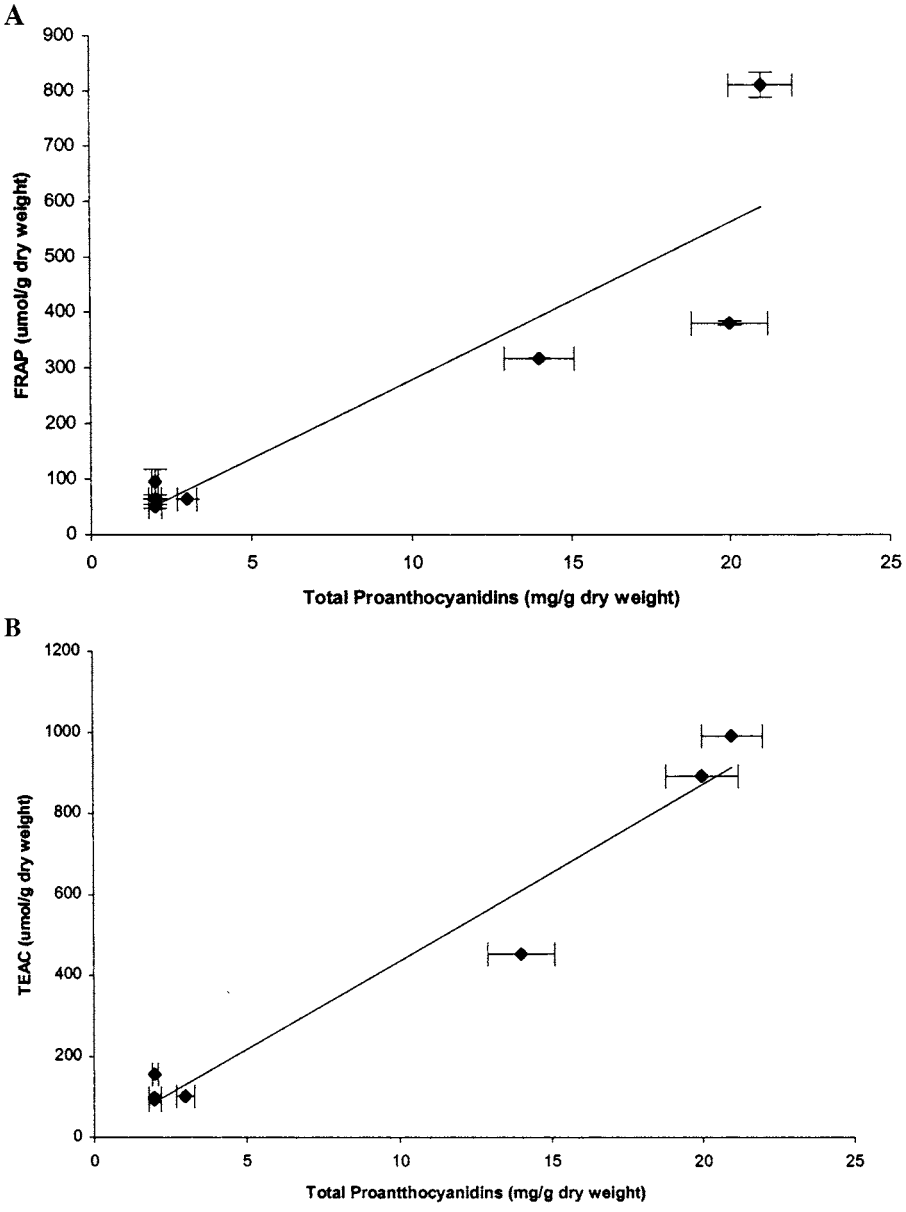

Figure 1. Effects of proanthocyanidins on (A) FRAP and (B) TEAC antioxidant capacities of *Cassia fistula*. Correlation coefficient between proanthocyanidins and antioxidant activities was assessed. For comparison with TEAC, $r = 0.980$; and for FRAP, $r = 0.899$.

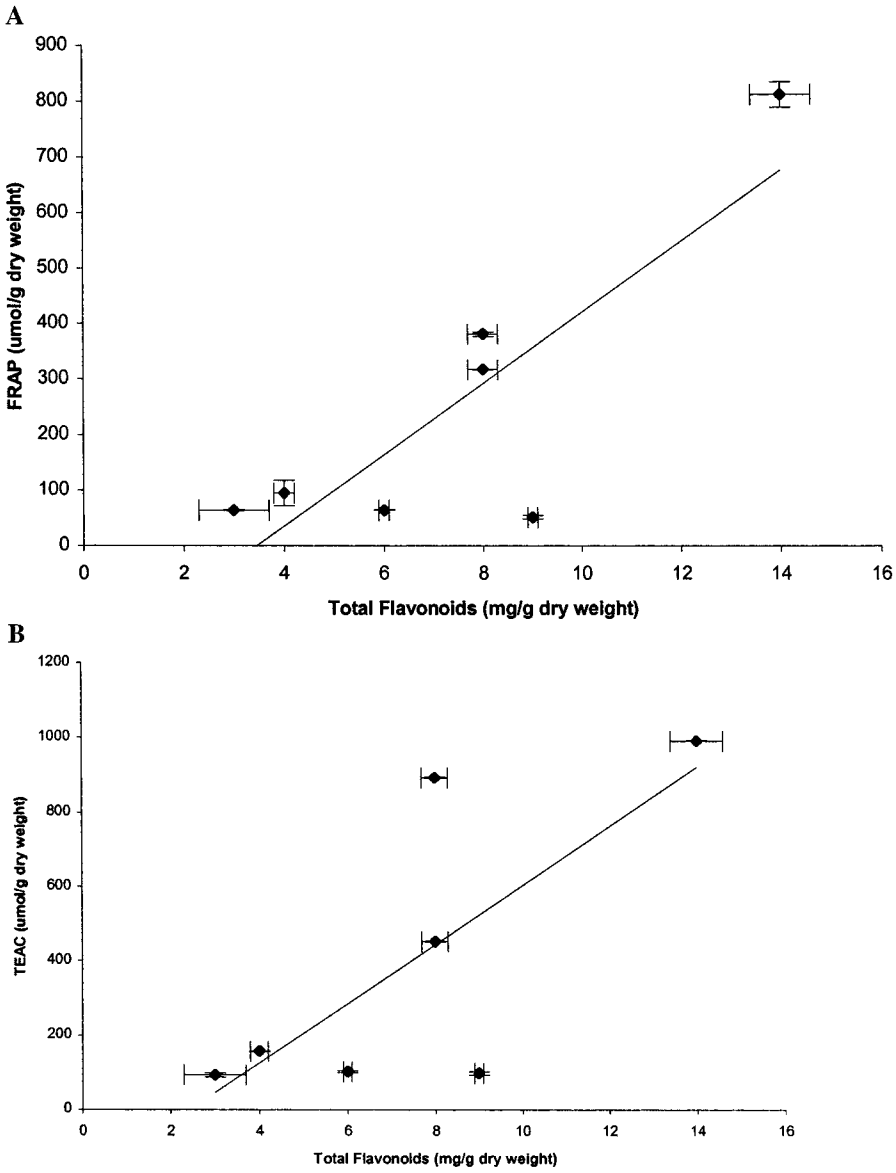

Figure 2. Effects of flavonoids on (A) FRAP and (B) TEAC antioxidant capacities of *Cassia fistula*. Correlation coefficient between flavonoids and antioxidant activities was assessed. For comparison with TEAC, $r = 0.737$; and for FRAP, $r = 0.835$.

453 ± 1.0 μmol/g dry weight), and the pods with the highest amount of polyphenols (54 ± 4.2 mg/g dry weight) showed the greatest antioxidant capacity (TEAC = 992 ± 0.4 μmol/g dry weight) (**Table 2**). The bark and twig extracts in vegetative organs and pod and flower extracts in reproductive organs also show good correlation ($r = 0.951$).

Total proanthocyanidins value of *Cassia fistula* showed good correlation with TEAC ($r = 0.980$) and FRAP ($r = 0.899$) (**Figure 1**). Proanthocyanidins seem to greatly influence the antioxidant capacity of reproductive organs, which in general produce high levels of these flavanol derivatives. Pods, containing the highest proanthocyanidin amounts with 21 ± 1.0 mg/g dry weight exhibited the greatest antioxidant property (TEAC 992 ± 0.4 μmol/g dry weight, FRAP 811 ± 23 μmol/g dry weight). Proanthocyanidin contents in vegetative parts range between 2 ± 0.1 and 3 ± 0.3 mg/g dry weight. The flavonoid contents of the extracts also showed good correlation with the antioxidant capacity of all *Cassia fistula* organs studied (TEAC $r = 0.737$, FRAP $r = 0.835$) (**Figure 2**). The flavonoid contents ranged between 3 ± 0.7 and 9 ± 0.1 mg/g dry weight in vegetative organs with the maximum amount produced by young leaves. Reproductive organs produce relatively higher quantities with amounts ranging between 8 ± 0.3 and 14 ± 1.3 mg/g dry weight, with optimum level produced by pods.

DISCUSSION

Natural and synthetic antioxidant compounds can exert a number of effects in vivo; e.g., promoting increased synthesis of endogenous antioxidant defenses or themselves acting directly as antioxidants. The feasibility of an extract or compound exerting antioxidant effects can be evaluated by in vitro tests that investigate how the putative antioxidant can or cannot react with relevant free radicals (*36*, *37*). Although the TEAC and FRAP assays are increasingly utilized to investigate antioxidant capacities of whole plant extracts (48), more particularly fruits (*14*, *49*), vegetables (*50*), and teas (*51*), concerns continue to be expressed about the need to consider factors such as colloidal properties of substrates, conditions of experimental methods, partitioning of antioxidants in substrates, and physiological relevance of the assays (*35*−*37*, *52*, *53*). In this study the antioxidant capacities of extracts from vegetative and reproduc-

tive organs of *Cassia fistula* were investigated. The extent of their antioxidant capacities were correlated with the contents of total phenolics, proanthocyanidins, and flavonoids. The best antioxidant activity and the highest levels of total phenolics, proanthocyanidins, and flavonoids were recorded in the pods, which coincidently is the harvest stage and organ recommended by the Pharmacopoeias. This is confirmed by linear regression analysis of the antioxidant activity with phenolic composition, which gave statistically significant correlations in all the organs studied (**Figures 1** and **2**). The pronounced antioxidant property observed in the reproductive organs can mainly be attributed to the high levels of proanthocyanidins (flavanol derivatives) comprising mainly catechins, and oligomeric and polymeric proanthocyanidins (*31*−*34*). Catechins and oligomeric proanthocyanidins have free radical scavenging and antilipoperoxidant activities (*42*, *54*−*56*) in various antioxidant assay systems. Catechins and kaempferol found in *Cassia fistula* (*33*, *34*) have good antioxidant index when compared with the antioxidant index of reference compounds (**Table 3**). On the basis of the TEAC and FRAP assays the flavan-3-ol derivatives seem to exhibit higher antioxidant capacities when compared with those of BHT, BHA, and ergothioneine. An association between the antioxidant potential of *Cassia fistula* extracts and the proportion of phenolics present as flavonoids was less evident despite the fact that these compounds are generally known to be potent antioxidants.

It thus appears that the important criteria for high-antioxidant *Cassia fistula* extracts are high total phenolics with high proanthocyanidin content probably necessitating the oxidative potency of flavonoids to add up to the synergistic overall antioxidant effect of the extracts.

Literature data abounds in reports where the same type of linear correlation between antioxidant activities and phenolic contents has been found in whole plant extracts, fruits, vegetables, and beverages. The antioxidant activities of extracts from vegetative and reproductive organs of *Crataegus monogyna* compared to total phenolics, proanthocyanidin, catechin, flavonoid, and phenolic acid contents is of great interest (*40*). The best correlations were established with total phenols, while activities in leaves seemed to be influenced by flavonoids, and in reproductive organs were influenced by proanthocyanidins and catechins (*40*). Deighton et al. (*49*) reported apparent linear relationships between antioxidant capacity (assessed as both TEAC and FRAP) and total phenols in a number of domesticated and wild *Rubus* species. Similar findings have been made while assessing the antioxidant activities of crude fruit extracts of *Ribes*, *Rubus*, and *Vaccinium* (*49*), medicinal plants (*57*), vegetables (*58*), teas (*51*, *59*), fruit juices (*60*), red wines (*61*), and fresh and processed edible seaweeds (*62*). This investigation shows the potential value of *Cassia fistula* extracts, notably the reproductive organs and fruit to be used as prophylactic antioxidant agents or to be utilized within existing programs for the eventual improvement of nutritional value of foods and their preservation. Further studies into the absorption and effects of *Cassia fistula* phenolics on antioxidant status in animal models are needed to evaluate their potential benefit.

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