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Studies on the antioxidant activity of Indian Laburnum (*Cassia fistula* L.): a preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp

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Abstract

The antioxidant properties of 90% ethanol extracts of leaves, and 90% methanol extracts of stem bark, pulp and flowers from Indian Laburnum (*Cassia fistula* L.) were investigated. The antioxidant activity power was in the decreasing order of stem bark, leaves, flowers and pulp and was well correlated with the total polyphenolic content of the extracts. The reason for low antioxidant activity in the flower and pulp fractions could be the presence of some prooxidants, such as chrysophanol and reducing sugars which dominate the antioxidant compounds present in the extracts. Thus, the stem bark had more antioxidant activity in terms of reducing power, inhibition of peroxidation, $O_2^{-\cdot}$ and DPPH radical scavenging ability.

Keywords: *Cassia fistula*; Antioxidant activity; Alcoholic extracts; Polyphenolics; DPPH$^{\cdot}$ radicals; Plant parts

1. Introduction

Currently there is much interest in the protection of low density lipoprotein and important cells and organs, as well as food systems, against oxidative damage caused by superoxide, hydroxyl and peroxyl radicals. Obviously, there has been an increasing demand to evaluate the antioxidant properties of direct plant extracts or isolated products from plant origin rather than looking for synthetic ones (McClements & Decker, 2000). It is an established fact that polyphenolic compounds, such as flavonoids, anthraquinones, anthocyanidins and xanthones, possess remarkable antioxidant activities which are present quite commonly in the plant family leguminosae. Recent works have highlighted the role of polyphenolic compounds of the higher plants (Hertog, Feskeens, Hollman, Katan, & Kromhout, 1993) such as flavonols (Salah, Miller, Pagana, Tijburg, Bolwell, & Rice-Evans, 1995), anthraquinones (Yen, Duh, & Chuang, 2000), xanthones and proanthocyanidins (Minami et al., 1994), which act as antioxidants or agents of other mechanisms that contribute to their anticarcinogenic or cardioprotective effects. Several studies have shown that increased dietary intake of natural phenolic antioxidants correlates with reduced coronary heart disease (Deshpande, Despande, & Salunkhe, 1996; Stampfer, Henneekens, Manson, Colditz, Rosner, & Willet, 1993).

Recent research investigations have suggested, that diets rich in polyphenolic compounds are associated with longer life expectancy (Hertog & Hollman, 1996). Moreover, these compounds have been found effective in many health-related properties, such as anticancer, antiviral, anti-inflammatory activities, effects on capillary fragility, and an ability to inhibit human platelet aggregation (Benavente-Garcia, Castillo, Marin, Ortuno, & Rio, 1997).

In our present study, a lesser-known legume, Indian Laburnum (*Cassia fistula*) has been used for the antioxidant studies since it is already known to be of good medicinal value. El-Saadany, El-Massry, Labib, and Sitohy (1991) reported that the seeds show hypocholesterolemic potential in hypercholesterolemic rats. Recently the hepatoprotective activity of leaf extract

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was reported by Bhakta, Banerjee, Mandal, Mait, Saha, and Pal (2001). Neverthless, the matured fruit pulp is consumed by rural people for a variety of reasons, such as a purgative, and to protect against heart disease and abdominal pain (Kirtikar & Basu, 1975). Moreover, the pulp has been reported to contain a high concentration of soluble sugars (sucrose, fructose and glucose) and is a rich source of macro-mineral elements, such as calcium and potassium (Barthakur, Arnold, & Alli, 1995). In addition, the chemical constituents of different parts of the plant have also been reported by various authors (Jawhar Lal & Gupta, 1972; Agrawal, Rizvi, Gupta, & Tewari, 1972; Meena Rani & Khalidhar, 1998; Vaishnav & Gupta, 1996; Misra, Singh, Pandey, & Singh, 1997; Gupta, Agarwal, & Tiwari, 1989; Kashiwada, Lizuka, Yoshioka, Chen, Nonaka, & Nishioka, 1990). However, information on the antioxidant activity or radical scavenging activity of different morphological parts of the plant is not available. In view of the above mentioned facts, reducing power, oxygen radical scavenging activity, peroxide inhibitory potential and DPPH· radical scavenging properties of aqueous alcoholic extracts of stem bark, leaves, flowers and pulp have been assessed.

2. Materials and methods

2.1. Materials and chemicals

The stem bark, leaves, flowers and mature fruit pulp of *C. fistula* L. were collected from Coimbatore, Tamil Nadu, India, during the month of November, 2000, shadow-dried at room temperature (25 °C) and then freeze-dried. The powder of the respective samples was stored at room temperature until subjected to further analysis. Riboflavin, linoleic acid, methionine, ascorbic acid, ammonium thiocyanate, butylated hydroxyanisole (BHA), thiobarbituric acid (TBA), nitro blue tetrazolium (NBT), egg lecithin and α,α-diphenyl-β-picrylhydrazyl (DPPH·) stable radical were purchased from Sigma Chemical Co. (St. Louis, MO). Trolox was procured from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Sodium dihydrogen phosphate, disodium hydrogen phosphate, trichloroacetic acid (TCA), ethanol and methanol were purchased from E. Merck Co. (Darmstadt, Germany).

2.2. Solvent extraction

The dried plant materials of stem bark, fruit pulp and flowers were extracted with 90% methanol in a Soxhlet apparatus for 16 h, whereas, the leaf materials were extracted with 90% ethanol in an aspirator bottle for 120 h. The solvents were evaporated in vacuo. The residues were freeze-dried and stored in a desiccator until further analysis.

2.3. Determination of total phenolic contents

The total phenolic content of the freeze dried samples of methanolic/ethanolic extracts was determined according to the method described by Makkar, Becker, Abel, and Pawelzik (1997). Suitable aliquots of the extract were taken in a test tube and made up to the volume of 1 ml with distilled water. Then, 0.5 ml of Folin-Ciocalteu reagent (1:1 with water) and 2.5 ml of sodium carbonate solution (20%) were added sequentially in each tube. Then the tubes were vortexed, placed in the dark for 40 min and the absorbance was recorded at 725 nm. The amount of total phenolics was calculated as tannic acid equivalents from the calibration curve.

2.4. Determination of antioxidant activity

The antioxidant activity of the extract, taken from different parts of the plant, was determined using the thiocyanate method (Yen & Hsieh, 1998). Each sample (5 mg in 1 ml of absolute ethanol) was combined with a mixture of linoleic acid (0.13 ml), 99.8% ethanol (10 ml) and 0.2 M potassium phosphate buffer (10 ml, pH 7) which was finally made up to 25 ml with water. The reaction mixture was incubated at 37 °C. Aliquots of 0.2 ml were taken at different intervals during incubation. The degree of oxidation was measured according to the thiocyanate method by sequentially adding ethanol (10 ml, 75%) ammonium thiocyanate (0.2 ml, 30%), sample solution (0.2 ml) and ferrous chloride (0.2 ml, 20 mM in 3.5% HCl). After the mixture was left for 3 min, the peroxide value was determined by reading the absorbance at 500 nm using a spectrophotometer (Hitachi, U-2000). A control was performed with linoleic acid, but without the extracts. Synthetic antioxidant, BHA, was used as a positive control.

2.5. Liposomes preparation and antioxidation evaluation

Liposomes were prepared according to the method of Tsuda, Ohshima, Kawakishi, and Osawa (1994), described by Yen et al. (2000). Egg lecithin (5 g) was dispersed in a sodium phosphate buffer (500 ml, 20 mM, pH 7.4) and sonicated in a sonicator (Bioblock Scientifica, Vibra cell) for 30 min under N_2 atmosphere in an ice-cold water bath. Varying concentrations (0.25, 0.50, 0.75, 1.0 and 1.50 mg ml^{-1}) of the extracts were tested for lipid peroxidation activities with the following mixture. The extract (0.5 ml) was mixed with liposomes (2 ml), 25 mM $FeCl_3$ (0.1 ml), 25 mM H_2O_2 (0.1 ml), 25 mM ascorbic acid (0.1 ml) and 0.2 M phosphate buffer (1.2 ml, pH 7.4). The reaction mixture was incubated at 37 °C for 4 h. At the end of the incubation, 1 ml of BHA (20 mg/ml in methanol) was added to stop the

oxidation reaction. The extent of oxidation of liposomes was subsequently determined by measuring the thiobarbituric acid-reactive substances (TBARS). To the reaction mixture was added 1 ml each of 1% thiobarbituric acid (TBA) and 10% HCl, and then it was heated in a water bath at 100 °C for 30 min. After the mixture was cooled in an ice bath for 15 min, 5 ml of chloroform were added, and the mixture was centrifuged at $3000\times g$ for 20 min. The absorbance of the supernatant was measured spectrophotometrically at 532 nm. Inhibition percent of TBARS formation was calculated as $100\times(A_0-A_t)/(A_0-A_1)$ where A_0, A_1, and A_t are the absorbance values for control, for blank, and for the test sample added, respectively. Trolox was assayed for comparison of the results.

2.6. Determination of the reducing power

The reducing power of the extracts was determined according to the method of Oyaizu (1986) as described by Yen et al. (2000). Lyophilised extract (2.5–15.0 mg) in 1 ml methanol was mixed with phosphate buffer (5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (5 ml, 1%) and the mixture was incubated at 50 °C for 20 min. Five millilitres of tricholoroacetic acid (10%) were added to the reaction mixture, which was then centrifuged at $3000\times g$ for 10 min. The upper layer of the solution (5 ml) was mixed with distilled water (5 ml) and ferric chloride (1 ml, 1%) and the absorbance was measured at 700 nm. Increased absorbance indicated increased reducing power.

2.7. Determination of the scavenging of superoxide radical

Superoxide radicals were generated by a modified method of Beauchamp and Fridovich (1971), described by Zhishen, Mengcheng, and Jianming (1999). All solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed in an aluminium foil-lined box with two 20 W fluorescent lamps. The distance between the reactant and the lamp was adjusted until the intensity of illumination reached about 4000 lx. The total volume of the reactant mixture was 5 ml and the concentrations of riboflavin, methionine and nitro blue tetrazolium (NBT) was 3×10^{-6}, 1×10^{-2} and 1×10^{-4} mol l^{-1}, respectively. The reactant was illuminated at 25 °C for 25 min. The photochemically reduced riboflavins generated $O_2^{-\cdot}$ which reduced NBT to form blue formazan. The unilluminated reaction mixture was used as a blank. Absorbance (A) was measured at 560 nm. The extracts were added to the reaction mixture, in which $O_2^{-\cdot}$ was scavenged, thereby inhibiting the NBT reduction. Absorbance (A_1) was measured and the decrease in $O_2^{-\cdot}$ was represented by A–A_1. The degree of the scavenging was calculated by the following equation: Scavenging $(\%)=(A-A_1/A)\times100\%$.

2.8. Scavenging effect on DPPH radical

The effect of extracts on DPPH· radical was estimated according to the procedure described by Moure, Franco, Sineiro, Domínguez, Nùnez, and Lema (2000). Two millilitres of a 3.6×10^{-5} M methanolic solution of DPPH· (Sigma) were added to 50 μl of a methanolic solution (1 mg ml^{-1}) of the antioxidant. The decrease in the absorbance at 515 nm was continuously recorded in a Hitachi U-2000 spectrophotometer for 16 min at room temperature. The scavenging effect (decrease of absorbance at 515 nm) was plotted against the time and the percentage of DPPH· radical scavenging ability of the sample was calculated from the absorbance value at the end of 16 min duration as follows:

$$\mathrm{IP}=\frac{(\text{absorbance}_t=0\text{ min}-\text{absorbance}_t=16\text{ min})}{\text{absorbance}_t=0\text{ min}}\times100$$

2.9. Statistical analysis

The data were subjected to a one-way analysis of variance (ANOVA) and the significance of the difference between means was determined by Duncan's multiple range test ($P<0.05$) using the Statistica for Windows H'97, Version 5.1 (Statsoft Inc., Tulsa, USA). Values expressed are mean ± S.D.

3. Results and discussion

3.1. The yield and total phenolic content of extracts

Table 1 shows the yield percentage and total phenolic content of extracts from different parts of the plant, *C. fistula*. Among the different extracts, the highest (33%) and the lowest (7.8%) yields of extraction were observed for the fruit pulp and leaves, respectively. However, the highest concentration of total phenolics (69.4%) was present in the stem bark extract, whereas the lowest concentration of phenolics (2.12%) was recorded in the pulp extract. Such a high yield potential of pulp extracts might be attributed to the presence of a high proportion of soluble sugars (54%; data not included).

The antioxidant activity of methanolic/ethanolic extracts of different parts of the plant exhibited the following order; stem bark > leaves > flowers > pulp, as established on the basis of the following experiments.

Table 1
Yield percent of solvent extracts and total phenolic constituents of different parts from *Cassia fistula*

Plant part	Yield %	Total phenolics of extract (g 100 g^{-1})[a]
Stem bark[b]	23.0	69.4
Leaves[c]	7.8	19.0
Pulp[d]	33.0	2.12
Flowers[e]	16.0	6.52

The solvent of the extract was removed under in vacuo, not exceeding more than 50 °C and extract was freeze-dried.

[a] Values are averages of two determinations.

[b] Sample (150 g) was extracted with 400 ml of methanol:water (9:1) in a Soxhlet apparatus for 16 h.

[c] Sample (1 kg) was extracted with 3 l of ethanol:water (9:1) in a aspirator bottle for 120 h.

[d] Sample (150 g) was extracted with 400 ml of methanol:water (9:1) in a Soxhlet apparatus for 16 h.

[e] Sample (30 g) was extracted with 400 ml of methanol: water (9:1) in a Soxhlet apparatus for 16 h.

3.2. Antioxidant activity of different extracts in the linoleic acid peroxidation system

From the results shown in Fig. 1 it was observed that almost all plant extracts (0.2 mg ml^{-1}) show a moderate activity between 37 and 62%. BHA (87.0%) was also compared, along with the extracts, at a concentration of 0.2 mg ml^{-1}. Thus, the peroxidation-inhibiting activity in the stem bark and leaf extracts was significantly ($P<0.05$) higher than that of the pulp extract. However, the antioxidant activity of all the extracts was significantly lower ($P<0.05$) than the reference BHA at the specified experimental concentrations. The presence of polyphenolic compounds, anthraquinones, xanthones, proanthocyanidins and flavonols, could be the reason for its reasonably good activity in the extracts of stem bark and leaves. Even though the presence of the antioxidants is proven in the extracts of pulp and flowers, the existence of prooxidant compounds might have suppressed the overall activity. In cases like stem bark and leaves, the presence of established antioxidants, such as xanthones, flavans, flavonols and di-anthraquinones are potentially responsible for its activity (Yen et al., 2000).

3.3. Reducing power

It has been cited in the reports (Yen & Duh, 1993) that the reducing power was associated with the antioxidant activity and this relationship was also established with the compounds of some anthraquinones (Yen et al., 2000). In our study we noticed that the reducing power was in the following order: stem bark > leaves > flowers > fruit pulp. Among all of the extracts, at the same concentrations, stem bark extracts showed a higher reducing power than the rest and leaves showed a moderate power. Flowers and pulp exhibited little reducing power (Fig. 2). According to Yen et al. (2000), some of the anthraquinones, which have also shown to possess antioxidant activity, showed no reducing power in their experiments. The same phenomenon occurs here for the relatively little reducing power in the pulp and flowers, even though pulp is reported to contain several anthraquinones and other polyphenols. Such relatively low effects might be due to the presence of prooxidants generated from the higher amounts of sugars, particularly mono and disaccharides existing in the pulp (Barthakur et al., 1995). Similarly, sugars such as pentoses, hexoses reducing disaccharides have been reported to be strong prooxidants of methyl linoleate and linoleic acid in aqueous emulsion systems (Mabrouk, 1964; Yamaguchi & Yamada, 1981; Yama-

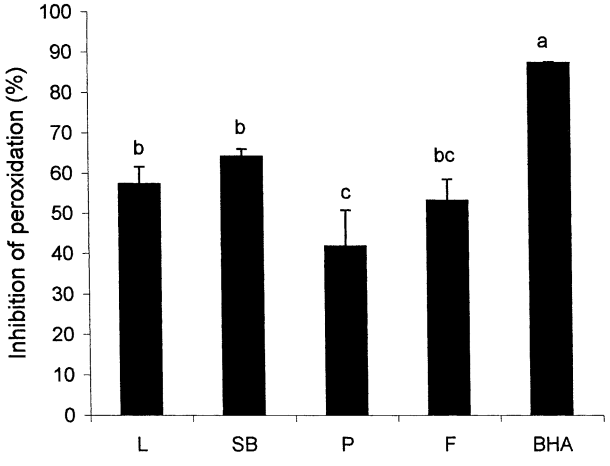

Fig. 1. Antioxidant activities of alcoholic extract (0.2 mg m^{-1}) of leaves (L), stem bark (SB), pulp (P) and flowers (F) from *C. fistula* and butylated hydroxyamsole (BHA). Values are means of three replicate analyses ± standard deviation. Values in each line with different letters are significantly different ($P<0.05$).

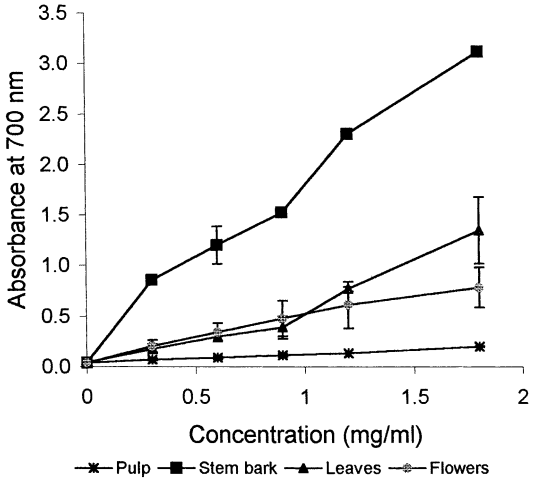

Fig. 2. Reducing power of total phenolic constituents of extract from different morphological parts of *C. fistula*. Values are means of two replicate analyses ± standard deviation.

guchi, Goto, Kato, & Ueno, 1984). On the other hand, chlorophylls are known to be well known photosensitisers, capable of converting triplet oxygen to singlet oxygen, and might also have acted as prooxidants and ultimately would have reduced the antioxidant capacity of the above mentioned extracts (Koryckа-Dahl & Richardson, 1978).

3.4. DPPH radical scavenging activity

The radical scavenging activity, using a DPPH generated radical, was tested with different sample extracts, along with BHT, and it was observed in the kinetics (Fig. 3) that the radical scavenging activity was very fast for the stem extracts, followed by leaves, but BHT, flowers and pulp exracts showed very slow kinetic behaviour. In terms of percentage, the inhibiting activity (at 16 min) was calculated to be in the following order; stem bark (93%), leaves (74.9%), BHT (37.8%), flowers (33.2%) and pulp (15.7%). These results reveal that stem bark and leaf extracts contain powerful inhibitor compounds, which may act as primary antioxidants that react with free radicals. The more rapidly the absorbance decreases, the more potent the antioxidant activity of the compound in terms of hydrogen donating ability (Von Gadow, Joubert, & Hansmann, 1997). The elevated DPPH· radical scavenging ability of the stem bark fraction might be due to the presence of two factors: (1) the high concentration of tannins (proanthocyanidins) (Kashiwada et al., 1990), and (2) flavonols and xanthones (Gupta et al., 1989). Similarly, Amarowicz, Naczk, and Shahidi (2000) reported that the tannins extracted from canola and rapeseed hulls exhibited a high scavenging efficiency toward DPPH· radicals. On the other hand, the presence of the 4′hydroxy configuration in the main compound of stem bark, flavan-3,4-diol (fistucacidin), could also be responsible for the hydrogen donating capacity (Jawahar Lal & Gupta, 1972). Moreover, the absence of the C2–C3 double bond and keto group does not necessarily account for the high DPPH· radical scavenging ability (Von Gadow et al., 1997).

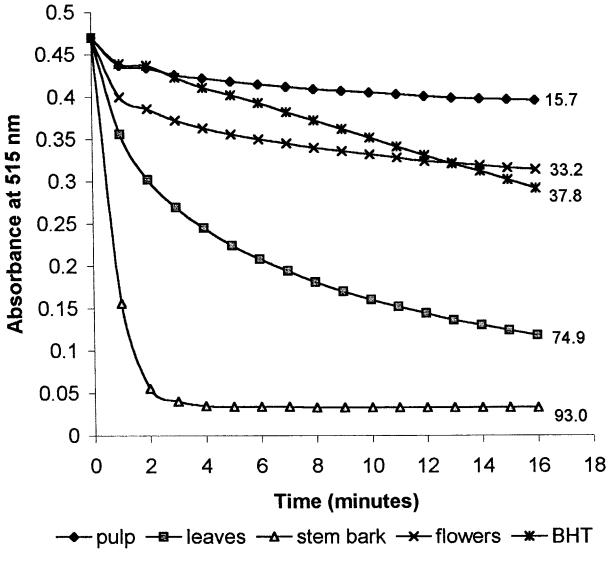

Fig. 3. α,α-Diphenyl-β-picrylhydrazyl (DPPH) radical-scavenging capacity of extract from different morphological parts of *C. fistula* and butylated hydroxytoluene (BHT). Values at the end of kinetic curves indicate the DPPH· radical-scavenging percent.

3.5. Antioxidant activity of extracts in the liposome peroxidation system

The behaviour of all the extracts was monitored after inducing radical peroxidation in egg lecithin and the inhibitory activity displayed by the stem is again noteworthy and comparable to trolox (91.7%) at high concentrations. The leaves showed moderate activity, but pulp and flower extracts did not exhibit any peroxidation inhibiting activity under the specified experimental concentrations. (data not shown in Fig. 4). In another report (Yen & Chuang, 2000), it was shown that trolox exhibited a similar good activity in a dose-dependent manner. These results indicate that some of the bioactive compounds, such as xanthones, flavonols and proanthocyanidins (Gupta et al., 1989; Kashiwada et al., 1990) present in the stem bark and leaf extracts, should perform as very good singlet oxygen quenchers since this is proven to be the precursor of the hydrogen peroxide and hydroxyl radical formation. It can also be predicted that some of the water-insoluble antioxidants, which can be incorporated into the nonpolar part of the liposome membrane, do effect antioxidant activity (Yen & Chuang, 2000).

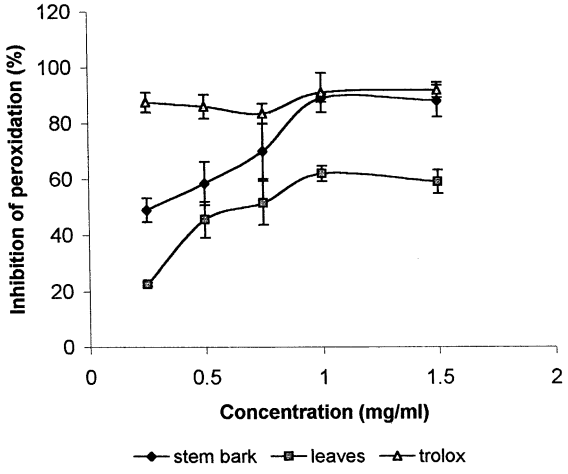

Fig. 4. Effect of alcoholic extract from different morphological parts of *C. fistula* on the lipid peroxidation of liposomes induced by Fe^{3+} H_2O_2/ascorbic acid.

Table 2
Superoxide radical scavenging ability of stem bark and leaf extracts from *Cassia fistula*

Sample concentration (ppm)	Superoxide radical scavenging percent	
	Stem bark	Leaves
4	38.5±13.4	1.82±0.54
8	59.5±10.6	6.0±2.83
16	77±15.6	24.0±2.83
20	89±1.4	27.5±2.12

Values are means of duplicate determinations±standard deviation.

3.6. Scavening ability of phenolics extracted from different parts of indian laburnum on superoxide radical

The superoxide radicals were generated by illuminating a solution containing riboflavin. The relative scavenging effects of extracts from different parts of *C. fistula* were assessed and the results are given in Table 2. Various concentrations of the sample (4, 8, 16 and 20 μg ml^{-1}) were used for the assay. Both flowers and pulp extracts appeared to have no scavenging activity on superoxide radicals. However, among the stem bark and leaves, extracts from the former exhibited the highest scavenging activity in a dose-dependent manner. This may be explained by the interaction of tannins, flavonols and flavans in the extracts. Moreover, it has also been reported that, in addition to tannins, (the flavonoid molecules with polyhydroxylated substitutions on rings A and B), a free 3-hydroxyl substitution would confer potent antisuperoxidative properties on the compounds (Zhishen et al., 1999). The main compound in *Cassia* stem bark, fistucacidin, contains a 4′ ortho-position hydroxyl which provides active hydrogen to take part in the following reaction to scavenge $O_2^{-\cdot}$

$$2H^+ + 2O_2^{-\cdot} = H_2O_2 + O_2$$

Similarly, the superoxide dismutase participates in the earlier reaction by catalysis, and as an antioxidant, through the supply of hydrogen. It seems that flavan-3,4-diol (fistucacidin) from *Cassia* stem bark scavenges $O_2^{-\cdot}$ in the same way.

4. Conclusions

Based on the active profile exposed through various assays, it can be concluded that, among the various plant fractions, the aqueous alcoholic extracts of stem bark and leaves show significant antioxidant activity, which may be accounted for by the high phenolic content (Table 1). The isolation of bioactive components in the extracts would certainly help to ascertain the individual potency of the compounds which could be further exploited for use by the food and pharmaceutical industries. No encouraging results were obtained in the flower and pulp fractions and, although there are reports of several antioxidant anthraquinones, it is concluded that the presence of considerable quantities of sugars such as glucose, fructose, reducing disaccharides, and sucrose, might have deactivated the antioxidant potential of those compounds present in the extract.

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