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Full Length Research Paper

Phytochemicals screening, DPPH free radical scavenging and xanthine oxidase inhibitiory activities of *Cassia fistula* seeds extract

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This study investigates antioxidant activity of *Cassia fistula* seeds extract. The antioxidant potential of this extract was evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay and xanthine oxidase inhibition assay. The IC_{50} values were calculated for DPPH and xanthine oxidase assays in order to evaluate the antioxidant efficiency of *C. fistula* seed extract. Total phenolic and flavonoid content were also determined. The seeds extract were exhibit antioxidant activity 59.587% with an IC_{50} value of 11.07 mg/ml in DPPH radical scavenging method. Furthermore, the *C. fistula* seeds extracts scavenged the superoxide radical generated by the xanthine/xanthine oxidase system. The percentage of xanthine oxidase inhibition activity of seed extract and allopurinol was 64.56 and 93.24%, respectively. The amount of total phenolic and flavonoid content was 474.25 ± 25.89 µg GAE/mg extract and 70.86 ± 1.43 µg catechin/mg extract respectively. High performance thin layer chromatography (HPTLC) screening indicates the presence of both free radical scavenging and phenolic compounds in *C. fistula* seed extract. The colored bands developed on TLC chromatogram visualized after spraying the plates with Folin-Ciocalteu's and DPPH reagents which represent phenolic and radical scavenging compounds, respectively.

Key words: *Cassia fistula* seed, antioxidant activity, xanthine oxidase inhibition, phenolic compounds.

INTRODUCTION

Oxidation is essential to many living organisms for the production of energy to fuel biological process. However, oxygen-centre free radicals and other reactive oxygen species (ROS), which are continuously, produced *in vivo,* results in cell death and tissues damage. Scientific evidence has suggested that under oxidative stress conditions, oxygen radicals such as superoxide anions (O_2^-), hydroxyl radical (OH) and peroxyl radicals (H_2O_2) are produced in biological system (Ramesh et al., 2009). These reactive oxygen species can damage DNA which causes mutation and chromosomal damage. It also oxidizes cellular thiols and extracts hydrogen atoms from unsaturated fatty acids to initiate the peroxidation of membranes lipids (Halliwell and Gutteridge, 1999; Ames et al., 1993). Moreover, the production of excessive free radicals stimulates the oxidative damage and such situation contribute to more than one hundred disorder in humans including atherosclerosis, coronary heart disease, neurodegenerative disorder, cancer and they playing major role in the aging process (Pong, 2003; Sandhya et al., 2010). Therefore, antioxidants are vital substances which possess the ability to protect the body from damage caused by free radicals induce oxidative stress (Ozsoy et al., 2008). This causes the possible toxicities of the synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hyroxytoluene (BHT), increasing attention has been directed towards natural antioxidants (Naimiki, 1990). There has been an upsurge

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of interest in the therapeutic potential of medicinal plants as antioxidants properties in reducing such free radical induced damages rather than looking for synthetic ones (McClements and Decker, 2000).

The search for plant-derived antioxidants has been received much attention and effort in order to identify the compounds that has high capacity in scavenging free radicals related to various diseases (Silva et al., 2007). Generally, antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide of lipid hydroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases. Epidemiologists have observed that a diet rich in polyphenolic compounds may result in a positive health effect attributed to its antioxidants properties (Frankel et al., 1996; Hertog et al., 1993). *Cassia fistula* L., a semi- wild Indian labernum, is widely cultivated as an ornamental tree for its beautiful bunches of yellow flowers and also traditional medicines for several ailments. It has also been used in traditional Brazilian medicines for the treatment of flu and cold, as a laxative and purgatives (Lorenzi and Matos, 2002).

There is documentation about the use of this *Cassia* sp. as a food source but there is still not much known about the antioxidant potential of *C. fistula* seeds. Therefore, the aim of the present study was to investigate and evaluate antioxidant activity and xanthine oxidase inhibitory effect of phenolic compounds from methanolic seeds extract of *C. fistula* and also to analyze flavonoids contents of the crude extract.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu reagents, 2,2-diphenyl-1-picrylhydrazyl (DPPH) reagent, gallic acid, allopurinol, aluminium trichloride (ACl_3), xanthine and xanthine oxidase, butylated hydroxytoluene (BHT) and catechin standards were purchased from Sigma-Aldrich (St. Louis, MO). Sulphric acid, phosphate buffer solution (pH 7.5), TLC Silica Gel 60 F_{253} alluminium sheet were was purchased from Merck (Darmatadt, Germany). Sodium nitrates (NaNO2), sodium hydroxide (NaOH), sodium carbonate (Na2CO3) were purchased from Fisher Scientific (Toronto, Ontario).

Plant collection

The pods of *C. fistula* were collected from various areas in Universiti Sains Malaysia, Penang in November 2009 and authenticated by the botanist of the School of Biological Sciences at Universiti Sains Malaysia where the herbarium was deposited. The sun-dried pods break and the seeds are separated. Then the seeds were washed under running tap water and dried in an oven at 50°C. Then the dried seeds homogenized to fine powder and stored in airtight bottles.

Solvent extraction

The powered plant material (200 g) was soaked in methanol (300 mL) for 7 days. The extract was filtered and the solvent was completely removed by a rotary evaporator. The extract was dried in the oven at 60°C to get a thick paste form. The crude extract were sealed in Petri plate and stored at room temperature for the assessment of antioxidant potential through various chemical assays.

2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

Quantitative measurements of radical scavenging assay were carried out according to the method described by Sasidharan et al. (2007). The quantitative measurement of radical scavenging properties was carried out in universal bottle. The reaction mixture contained 50 µl of seeds extract at concentration ranging from 0.031 to 1 mg/ml and 5 ml of a 0.04% (w/v) solution of DPPH in 80% methanol. The commercial known antioxidant, butylated hydroxytoluene (BHT) was used for comparison or as a positive control. The DPPH solution in the absence of seeds extract was used as control and the 80% methanol was used as blank. Discolourations were measured at 517 nm by using spectrophotometer (HITACHI U-1900 spectrophotometer 200V) after incubation for 30 min in the darkroom. Measurement was performed at least in triplicate. The percentage of the DPPH free radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = ((A_0 - A_1)/ A_0) \times 100$$

Where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the seeds extract of *C. fistula* (Oktay et al., 2003). The actual decrease in absorption induced by the test was compared with the positive controls. The IC_{50} (concentration providing 50% inhibition) values were calculated use the dose inhibition curve in linear range by plotting the extract concentration versus the corresponding scavenging effect.

Total phenolic content

The total phenolic content in the methanol seeds extract of *C. fistula* was measured using Folin-Ciocalteu reagent based on procedures described by Singleton et al. (1999), with some modifications. Briefly, 0.5 ml of methanolic seeds extract (1 mg/ml) was mixed with 1.5 ml (1:10 v/v diluted with distilled water) Folin-Ciocalteau's reagent and allowed to stand for 22°C for 5 min. Then 2 ml of sodium carbonate (Na_2CO_3, 7.5%, w/v) was added and the mixture were allowed stand for another 90 min and kept in the dark with intermittent shaking. Then the absorbance of the blue colour that developed was measured at 725 nm using spectrophotometer (HITACHI U-1900 spectrophotometer 200V). The experiment was carried out in triplicates.

Gallic acid was used for constructing the standard curve (25 to 150 µg/ml; Y= 0.0008X; R^2= 0.9876) and the total phenolic compounds concentration in the seeds extract was expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g) of extract.

Flavonoid content

Total flavonoid content of the extract was determined according to colorimetric method described by Zhishen et al. (1999), with some modification. Briefly 0.5 ml seeds extract (1 mg/ml) was added in three bijoux bottles and mixed with 2 ml of distilled water. Subsequently add 0.15 ml of sodium nitrite ($NaNO_2$, 5% w/v) into each bottles and the reaction mixture was allowed to stand for 6 min. Then 0.15 ml aluminium trichloride ($AlCl_3$, 10%) was added

and allowed to stand for 6 min, followed by addition of 2 ml of sodium hydroxide (NaOH, 4% w/v) to the reaction mixture. Then distilled water was added to the mixture to bring the final volume up to 5 ml. the reaction mixture was mixed thoroughly and allowed to stand for another 15 min. Then absorbance of pink colour that developed was measured at 510 nm using spectrophotometer (HITACHI U-1900 spectrophotometer). Distilled water was used as blank.

The final absorbance of each sample was compared with a standard curve plotted from catechin. The total flavonoid content was expressed in mg of catechin per gram of extract.

Xanthine oxidase assay

The xanthine oxidase (XO) activities with xanthine as the substrate were measured spectrophotometrically using the procedure of Marcocci et al. (1994). The xanthine solution (150 mM) was prepared by initially dissolving xanthine in phosphate buffer solution (PBS), and adjusting the pH to 7.5. The XO solution was prepared in cold 50 mM phosphate buffer (pH 7.5) at concentration of 0.4 U/ml. The extract was serially diluted to achieve a concentration range of 0.031 to 1 mg/ml. The assay mixture was prepared consisting of 1 ml of extract from each concentration, 2.9 ml of phosphate buffer solution (pH 7.5) and 2 ml of xanthine solution. This mixture was pre-incubated at 25°C for 15 min. The reaction was initiated by adding 0.1 ml of XO solution and the sample were incubated at 25°C for 30 min.

The reaction was stopped by addition of 1N hydrochloric acid (HCl) and the absorbance was read at 295 nm using UV-spectrophotometer. The buffer solution was used as blank and solution containing xanthine and xanthine oxidase was used as control. Allopurinol is a known inhibitor of xanthine oxidase and was used as positive control. The inhibition percentage of xanthine oxidase activity was calculated according to the formula:

$$\text{Inhibition (\%)} = \frac{(A - B) - (C - D)}{A - B} \times 100$$

Where *A*; the activity of the enzyme without test extract, *B*; control of *A* without test extract and enzyme, *C* and *D*; the activities of the test extract with and without XO respectively. IC_{50} values were calculated from the mean values of data.

High performance thin layer chromatography (HPTLC) study of phenol and DPPH

Methanolic seed extract of *C. fistula* was subjected to High performance thin-layer chromatography (HPTLC) on silica gel plate (20x 20 cm, Silica gel F_{254}, Merck). The solvent system optimized for crude extract of *C. fistula* was methanol and Chloroform (10: 90 v/v). The seeds extract were load on TLC silica gel plate and the plate were developed in a sandwich TLC chamber to a distance of 70 mm. After 15 min air-drying, the plate was sprayed with 0.004 % (w/v) DPPH reagent prepared in methanol, Folin-Ciocalteu's reagent (1:10 v/v in distilled water) and 10% (v/v) sulpric acids respectively. The spots on the plates were observed after the plate has been heated at 60°C for 30 min exactly after spraying.

Statistical analysis

All the analysis were carried out in triplicate and expressed as mean ± SD. Analyses of variance were performed using the one-way analysis of variance (ANOVA). Significant differences between means were determined by Duncan's multiple range tests. P values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

DPPH scavenging assay

Antioxidant properties of methanol seeds extract of *C. fistula* were evaluated to find a new natural source of antioxidant. DPPH radical is a commonly used substrate for fast evaluation of antioxidant activity because of its stability in the radical form and simplicity of the assay (Bozin et al., 2008). This assay is known to give reliable information concerning the antioxidant ability of the tested compounds (Huang et al., 2005). The principle behind this assay in the color change of DPPH solution from purple to yellow as the radical is quenched by the antioxidant (Karagözler et al., 2008). The colour changes can be measured quantitatively by spectrophotometer absorbance at 517 nm.

The seeds extract of *C. fistula* was screed for DPPH radical scavenging activity according to the method described and the results of the screening are shown in Figure 1 as comparable with BHT, known antioxidant. The methanolic extract show lower reducing power activity than BHT. The reducing power activity of BHT was 65.7% followed by the seeds extract 46.6% at the concentration tested (1 mg/ml). Therefore, such phenomena suggest that the seeds extract of *C. fistula* may act as free radicals scavenger and may react with radicals to convert them to more stable products and terminate radical chain reaction (Duh and Yen, 1997).

Moreover, based on the demonstrated results the crude extract of *C. fistula* can be categories as moderate free radical scavenger. Apart from that, the methanolic seeds extract exhibit a significant dose dependent inhibition of DPPH activity with 50% of inhibition (IC_{50}) at concentration of 11.07 mg/ml (Figure 1a) and IC_{50} value of BHT was 7.76 mg/ml (Figure 1b). Basically, a higher DPPH radical-scavenging activity is associated with a lower IC_{50} value. There are studies have been carried out to evaluate the antioxidant activity of *Cassia* species using DPPH assay (Sangetha et al., 2008) and reported that, particularly *C. fistula* exhibited higher antioxidant activity compared to *C. spectabilis* sp. Whereby the present study proof that, the seeds extract of *C. fistula* has the potential compound(s) react as antioxidant which is suitable to develop a drugs for the prevention of human disease related to free radical mechanism.

Total phenolic and flavonoids content

Plant phenolic constitutes one of the major groups of compounds acting as primary antioxidants or free terminators (Agarwal, 1989). Phenolic compounds are commonly found in both edible and inedible plants, and have been reported to have multiple biological effects, including antioxidant activity. Phenolics are able to scavenge reactive oxygen species due to their electron

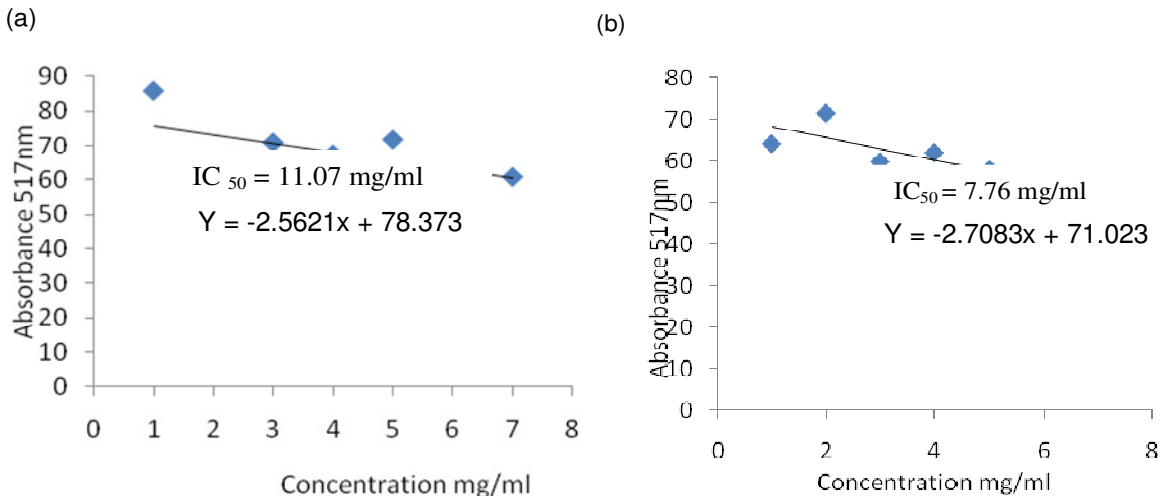


Figure 1. The IC_{50} value obtained by linear regression equation for (a) seeds extract of *C. fistula* and (b) BHT.

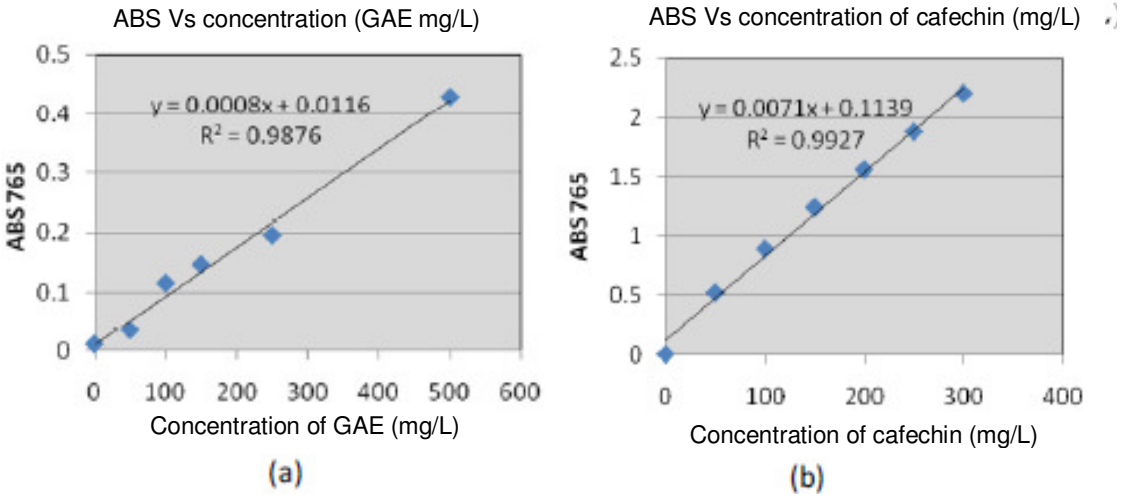


Figure 2. Standard curve for determination of (a) Gallic acid and (b) Catechin Equivalents for total phenolic and flavonoids assay.

donating properties. Their antioxidant effectiveness depends on the stability in different systems, as well as number and location of hydroxyl groups (Pods-edek, 2007). The phenolic compounds such a phenolic acid and flavonoids are most important antioxidant food source. The quantitative analysis of phenolic acids and flavonoids by measurement UV absorption is well known (Jurd and Geissmao, 1956). In the present study, the total phenolic and flavonoids content of the *C. fistula* seeds extract were analyzed.

The total phenolic content was determined using Folin-Ciocalteu method, reported as gallic acid equivalents by reference to standard curve Figure 2a (y = 0.0008x + 0.0116 and r^2 = 0.9876). The total phenolic content extract was 474.25 ± 25.98 mg GAE/g of extract (Table 1). Whereby the flavonoids content was 70.86 ± 1.43 mg of catechin equivalent per gram of sample by reference to standard curve (y = 0.0071x + 0.1139 and r^2 = 0.9927) (Figure 2b). Basically, phenols and polyphenolic compounds, such as flavonoids are widely found in seeds extract of *C. fistula* may react as antioxidant observed in this study.

Xanthine oxidase inhibition assay

The xanthine oxidase inhibition activity was evaluated using the above method. The result was shown in Figure 3. The xanthine oxidase inhibition activity of *C. fistula* seed extract was 64.56% ± 0.4. Allopurinol, a known

Table 1. Total phenolic contents of seeds extract of *Cassia fistula*.

Sample	Total phenolic contents (µg/ mg) GEA
Seeds	474.25 ± 25.98

Values are expressed as means ± SD; P< 0.05. GAE (gallic acid equivalents).

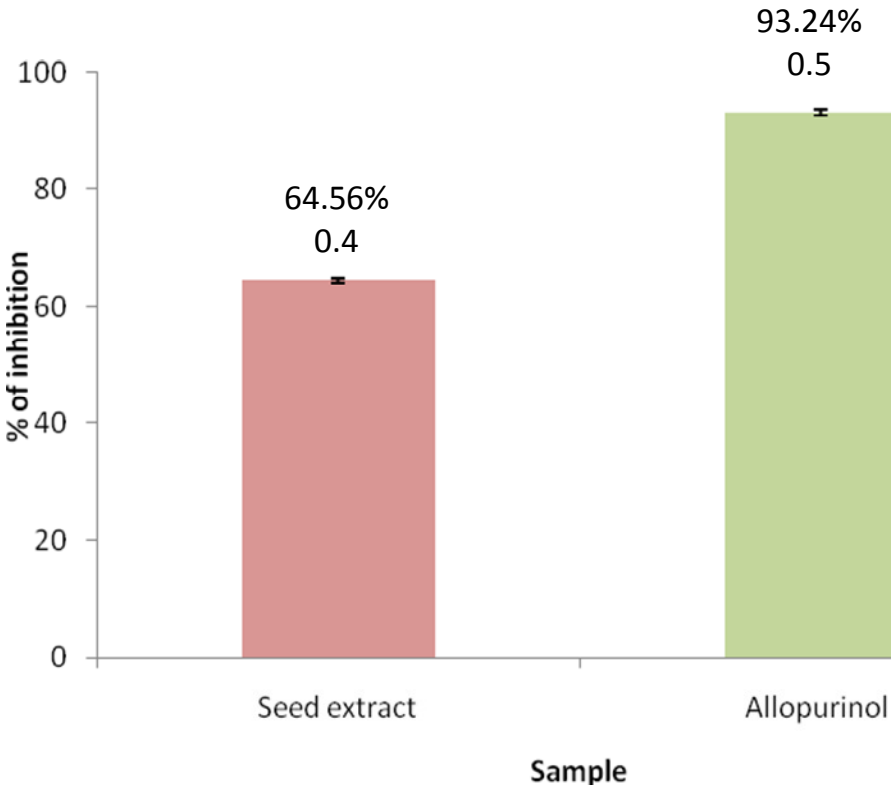


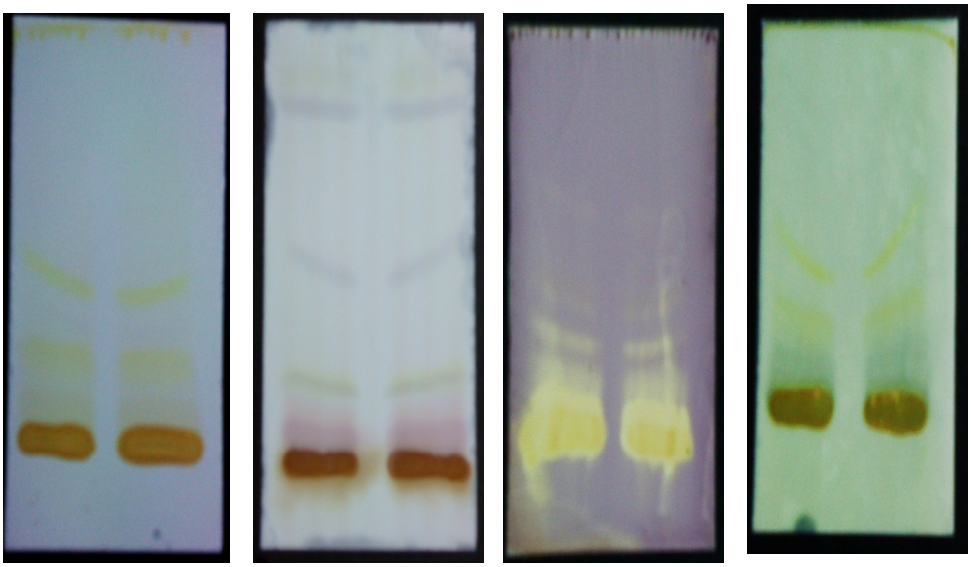
Figure 3. Xanthine oxidase inhibition (%) of seed extract of *C. fistula* and known antioxidant allopurinol, 100 µg/ml (P> 0.05).

inhibitor of Xanthine oxidase which was used as positive control indicated a high inhibitory value, 93.25% ± 0.5. Xanthine oxidase has been reported as a very important enzyme that increases during oxidative stress. Two major disease related to xanthine oxidase (XO) are gout and hyperuricemia. Gout is a clinical metabolism disorder, which is associated with increased level of uric acid in blood (Liu et al., 2008). Compounds that enhance the excretion of uric acid or inhibit uric acid biosynthesis, or have anti-inflammatory actions are generally used for treatment of gout.

In this study, seeds extract of *C. fistula* exhibited xanthine oxidase inhibition activity. This particular extract which inhibits XO could be very beneficial in treating the above mention diseases. Hence, from this study seed extract of *C. fistula* could be continuously study further to develop XO inhibition agent.

HPTLC bioautography analysis of phenolic and antioxidant substance

Bioautography technique is a very useful method to detect the presence of phenolic and antioxidant substance in plant extracts. In this study, HPTLC bioautography was used to detect the phenolic and antioxidant substances in *C. fistula* seeds extract as shown in Figure 4. Figures 4b, c and d show the profile of separated bands after spraying with 10% sulphric acid, 0.004% DPPH reagent and Folin-Ciocalteu reagent respectively. One purple and 2 yellow colour spots (Figure 4b) were visualized after spraying with sulphric acid demonstrated the presence several bioactive compounds. Three yellow spots (Figure 4c) on purple background were observed those indicating, the presence of antioxidant compounds. In addition, plate



(a) (b) (c) (d)

Figure 4. Phenol and antioxidant substance of *C. fistula* seed extract in developed TLC chromatogram (methanol: chloroform, 10: 90 v/v). (a) Separation of bands, (b) Coloured bands after spraying with 10% sulphric acid, (c) Pale- yellow radical scavenging bands on purple colour background after spraying with 0.04% v/v DPPH reagent, and (d) blue phenolic bands on yellow background after spraying with Folin-Ciocalteu reagent.

stain with Folin-Ciocalteu reagent demonstrated one active blue colour band (Figure 4d) with yellow background indicating the presence of phenolic compound.

In conclusion, the HPTLC bioautography method revealed that the antioxidant activity observed in this study probably contribute by phenolic substances in the seeds extract.

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