HPLC ANALYSIS AND ANTIOXIDANT POTENTIAL OF PLANT EXTRACT OF CASSIA ALATA

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ABSTRACT
The recent studies have investigated the antioxidant effect of plant Cassia alata and activity mainly attributed to phenolic compounds, such as flavonoids, phenolic acids, tannins etc. Studies have been taken up to evaluate the antioxidant potentials in plant extract by various means. Antioxidant potential was found to be more in extract as compared to control. Further, studies were also conducted for the identification of phenolic compounds with different solvents using Reverse Phase High Pressure Liquid Chromatography (RP-HPLC) coupled with photodiode array detector. The results of this study clearly indicated that the extracts of Cassia alata could be used as a potential source of natural antioxidant agents.

Key words: Cassia alata, RP-HPLC, Antioxidant Potential, Flavonoids, Phenolic Contents.

INTRODUCTION
Polyphenolic compounds tend to be potent free radical scavengers and their abilities to act as antioxidants mainly depends on their chemical structure, capability to donate/accept electrons, thus delocalizing the unpaired electron within the aromatic structure and the polyphenols are broadly classified into two categories, flavonoids and phenolic acids [1]. Flavonoids is a large family consisting of more than 4000 ubiquitous secondary plant metabolites, which are further divided into 5 subclasses namely flavonols, flavones, anthocyanins, catechins and flavonones [2]. Polyphenols like, Caffeic acid act as inhibitor for lipoxygenase enzyme, suppress acute immune and inflammatory response and known to have antimitogenic, anticarcinogenic and immunomodulatory properties. The antioxidant properties of polyphenols depend mainly upon factors such as metal reducing potential, chelating behavior, pH and solubility characteristics [3, 4]. Cassia alata is categorized under the family Fabaceae, a pan tropical ornamental shrub, 2-3m high, widely distributed in tropical countries, stretching from Tropical America to India, Fiji, Indonesia, Malaysia and Africa [5]. Many reports have shown that some Cassia species contain antimicrobial substances, particularly Cassia alata [6, 7]. Cassia alata showed the presence of phenol, tannins, anthraquinones, saponins and flavonoids, the plant also had alkaloids and cardenolides [8].

The main objectives of present study were to evaluate antioxidants potential of Cassia alata and HPLC analysis of major constituents.

MATERIALS AND METHODS
Plant material
Collected plant material were cleaned, dried under shade at room temperature then grounded and extracted. All chemicals and reagents were of analytical grade.

Preparation of the plant extract
The air-dried plant material was reduced up to optimum size by grinding and extracted with methanol using a Soxhlet apparatus. The mixture was filtered through filter paper (Whatman, No. 1) and evaporated. The residue was stored in a dark glass bottle for further processing.

Determination of total phenolic content
Total phenols were estimated according to the Folin–Ciocalteu method [9]. The extract was diluted to the concentration of 1 mg/ml, and aliquots of 0.5 ml were mixed with 2.5 ml of Folin Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 ml of NaHCO3 (7.5%). After 15 min at 45°C, the absorbance was measured at 765 nm using a spectrophotometer against a blank.

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sample. Total phenols were determined as gallic acid equivalents (mg GA/g extract).

**Determination of flavonoid content**

Total flavonoids were determined according to known procedure [10]. A total of 0.5 ml of 2% aluminum chloride (AlCl₃) in methanol was mixed with the same volume of methanol solution of plant extract. After 1 h of staying at room temperature, the absorbance was measured at 415 nm in a spectrophotometer against the blank sample. Total flavonoids were determined as rutin equivalents (mg RU/g dry extract).

**HPLC Analysis**

High-performance liquid chromatography (HPLC) analysis was carried out with: C₁₈ column (C₁₈; 25 cm×4.6 mm, 10 μm) Acetonitrile–water—phosphoric acid (85:15:0.1, v/v/v) was used as the mobile phase for HPLC analysis because it provides excellent separation of peaks in chromatograms. Flavonoid was identified on the base of its retention time and absorption spectrum and by comparison of these results with those of standard.

**Antioxidant activity**

**DPPH Assay**

DPPH (2,2-diphenyl-1-picrylhydrazyl) (8 mg) was dissolved in methanol (100 ml) to obtain a concentration of 80 μg/ml. Serial dilutions were carried out with the stock solution (1 mg/ml) of the extract. Solutions (2 ml each) were then mixed with DPPH (2 ml) and allowed to stand for 30 min for any reaction to occur, and the absorbance was measured at 517 nm. Ascorbic acid, gallic acid (GA) and butylated hydroxytoluene (BHT) were used as reference standards and dissolved in methanol to make the stock solutions with the same concentration (1 mg/ml). Control sample was prepared containing the same volume without test compounds or reference antioxidants. 95% Methanol was used as a blank. The DPPH free radical scavenging activity (%) was calculated using the following equation; where Ac is the absorbance of control solution and As is the absorbance of the sample solution: 

\[
\text{Inhibition} = \frac{\text{As} - \text{Ac}}{\text{Ac}} 
\]

The IC₅₀ value, defined as the concentration of the test material that leads to 50% reduction in the free radical concentration, was calculated as μg/ml through a sigmoidal dose-response curve.

**Determination of inhibitory activity against lipid peroxidation**

Antioxidant activity was determined by the thiocyanate method. Serial dilutions were carried out with the stock solution (1 mg/ml) of the extracts, and 0.5 ml of each solution was added to linoleic acid emulsion (2.5 ml, 40 mM, pH 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g linoleic acid, 0.2804 g Tween-20 as emulsifier in 50 ml 40 mM phosphate buffer and the mixture was then homogenized. The final volume was adjusted to 5 ml with 40 mM phosphate buffer, pH 7.0. After incubation at 37 °C in the dark for 72 h, a 0.1 ml aliquot of the reaction solution was mixed with 4.7 ml of ethanol (75%), 0.1 ml FeCl₂ (20 mM) and 0.1 ml ammonium thiocyanate (30%). The absorbance of the mixture was measured at 500 nm and the mixture was stirred for 3 min. Ascorbic acid, gallic acid, α-tocopherol and BHT were used as reference compounds. To eliminate the solvent effect, the control sample, which contained the same amount of solvent added to the linoleic acid emulsion in the test sample and reference compound, was used. Inhibition percent of linoleic acid peroxidation was calculated using following equation; where Ac is the absorbance of control solution and As is the absorbance of the sample solution:

\[
\text{Inhibition} = 100(\text{Ac} - \text{As})/\text{Ac}
\]

**Determination of hydroxyl radical scavenging activity**

The reaction mixture contained 100 μl of extract dissolved in water, 500 μl of 5.6 mM 2-deoxy-D-ribose in KH₂PO₄-NaOH buffer (50 mM, pH 7.4), 200 μl of premixed 100 μM FeCl₂ and 104 mM EDTA (1:1, v/v) solution, 100 μl of 1.0 mM H₂O₂ and 100 μL of 1.0 mM aqueous ascorbic acid. Tubes were vortexed and incubated at 50 °C for 30 min. Thereafter, 1 ml of 2.8% TCA and 1 ml of 1.0% TBA were added to each tube. The samples were vortexed and heated in a water bath at 50°C for 30 min. The extent of oxidation of 2-deoxyribose was estimated from the absorbance of the solution at 532 nm. The percentage inhibition values were calculated from the absorbance of the control (Ac) and the sample (As), where the controls contained all the reaction reagents except the extract or positive control substance.

**RESULTS AND DISCUSSION**

**HPLC Analysis**

Figure 1 shows HPLC chromatograms of the methanol extract Acetonitrile–water—phosphoric acid (85:15:0.1, v/v/v) was used as the mobile phase for HPLC analysis because it provides excellent separation of peaks in chromatograms. Flavonoid presence was confirmed by comparing of the retention time with the standard.

**Antioxidant activity**

Phenolic compounds and flavonoids have been reported to be associated with antioxidant action in biological systems, mainly due to their redox properties, which can play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. One of the more prominent properties of flavonoids is their excellent radical scavenging ability. It is also a valuable aspect for therapeutic and prophylactic applications of flavonoids. Total phenolic contents were determined and amounted to
74.35±0.89. The contents of flavonoids were determined and amounted to 24.37±0.25 (Table 1). Antioxidant activity of the methanol extracts was evaluated using the DPPH, hydroxyl radical scavenging and lipid peroxidation assays. The results (Table 1) showed that extracts possess capacity of 71.35±0.32 μg AA/g and IC₅₀ values of 23.69±0.84 μg/ml for DPPH free radical scavenging activity (Figure 2), 38.17±1.2 μg/ml for inhibitory activity against lipid peroxidation and 95.46±0.79 μg/ml for hydroxyl radical scavenging activity.

Table 1. Antioxidant activity and total phenolic/flavonoids contents of extract

<table>
<thead>
<tr>
<th>Antioxidant activity of extract</th>
<th>Total phenolic and flavonoids</th>
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<tbody>
<tr>
<td>DPPH scavenging activity</td>
<td>71.35±0.32</td>
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<tr>
<td>Inhibitory activity against lipid peroxidation</td>
<td>38.17±1.2</td>
</tr>
<tr>
<td>Hydroxyl radical scavenging activity</td>
<td>95.46±0.79</td>
</tr>
<tr>
<td>Total phenolic</td>
<td>74.35±0.89</td>
</tr>
<tr>
<td>Total flavonoids</td>
<td>24.37±0.25</td>
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Figure 1. HPLC Chromatograms of the methanol extract (A) and standard (B).

Figure 2. Result of DPPH free radical scavenging activity of extract

CONCLUSION

Finding new antioxidant sources could be important for health benefits considering many diseases that reactive oxygen species induce in biological systems. Discovering a natural source of antioxidants could also be significant for artificial toxic antioxidants replacement in food industry. The results of this study clearly indicated that methanol plant extracts are good scavengers of synthetic DPPH radicals, indicating that they could be used as antioxidant products. Also, they all possess reductive capabilities. The reductive capacities of the investigated extracts are lower than those of the investigated standard antioxidant compounds, so they could be considered primarily as antioxidants and then as products with reductive capabilities. The activity of tested extract is probably derived from a coumarin derivative, a compound that has previously been found to possess various types of biological activity. The results provided evidence that the studied plant might indeed be a potential source of natural antioxidant agents.

REFERENCES


