DETERMINATION OF TOTAL FLAVONOID CONTENT, TOTAL POLYPHENOL CONTENT AND ANTIOXIDANT ACTIVITIES OF *Holarrhena pubescens*

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**ABSTRACT**

Traditionally *Holarrhena pubescens* has been used as analgesic, antilithiasic, and antiseptic. It is used for the treatment of edema as well as for cystitis, gout, kidney stones, nephritis, and prostatitis. In the present study, the antioxidant properties of ethanol-water extract from *H. pubescens* were estimated by different methods. Also phenol and flavonoid content of the extract were measured by Folin Ciocalteu and AlCl₃ assays. *H. pubescens* extract contained a significant amount of phenol and flavonoids. The percentage of DPPH radical scavenged by extract was 92.6 at a concentration of 1.6 mg ml⁻¹. IC₅₀ of the extract and the standard compounds butylatedhydroxytoluene (BHA) and quercetin was 0.59, 0.053, and 0.025 mg ml⁻¹, respectively. Iron chelating activity of the extract was less than the standard compounds. *H. pubescens* extract showed nitric oxide-scavenging effect less than the reference agent (quercetin). The extract showed a high reducing ability. According to ferric thiocyanate (FTC) method, the extract showed more than 88% inhibition of linoleic acid peroxidation. It might be concluded that some of the properties of *H. pubescens* in traditional medicine is due to its antioxidant ability.
INTRODUCTION
The potentially reactive derivatives of oxygen, attributed as reactive oxygen species (ROS), are continuously generated inside the human body as a result of contact with excess of exogenous chemicals in our ambient environment and/or due to a number of endogenous metabolic processes involving redox enzymes. Under normal circumstances, the ROS generated are detoxified by the antioxidants present in the body and there is equilibrium between the ROS generated and the antioxidants present. However, owing to ROS overproduction and/or inadequate antioxidant defense, this equilibrium is interfered favoring the ROS upsurge that terminates in oxidative stress. The ROS easily affect and persuade oxidative damage to various biomolecules including proteins, lipids, lipoproteins, and DNA. This oxidative damage is a critical etiological factor implicated in several chronic human diseases such as diabetes mellitus, cancer, atherosclerosis, arthritis, and neurodegenerative diseases and also in the ageing process. Based on the growing interest in free radical biology and the lack of effective therapies for most chronic diseases, the usefulness of antioxidants in protection against these diseases is supported. Epidemiological studies have found that the intake of antioxidants, such as Vitamin C, reduce the risk of coronary heart disease and cancer. The antioxidants may mediate their effect by directly reacting with ROS, quenching them and/or chelating the catalytic metal ions. Several synthetic antioxidants, such as butylatedhydroxyanisole and butylatedhydroxytoluene, are commercially available but are quite unsafe and their toxicity is a problem of concern. Especially phenolic and flavonoid compounds derived from plants were proved to be potent antioxidant and free radical scavengers. Therefore, in recent years, considerable attention has been directed towards the identification of plants with antioxidant ability that may be used for human consumption. Many plants have been investigated in the search for novel antioxidants. The present study focuses on determination of total flavonoid content, total phenol content and determination of antioxidant activity of H. pubescens.

MATERIAL AND METHOD
Chemicals
Gallic acid, DPPH, quercetin, BHA, BHT, Vitamin C, and EDTA were purchased from Merck and Flukacompanies. All other chemicals and reagents used were of the highest commercially available purity.
Preparation of extracts of *H. pubescens*

*H. pubescens* used for this investigation was collected in March 2012 and authenticated in by Mr. Ahmed Nikar and a voucher specimen, number 435/01AF, was deposited at the herbarium section of the faculty. The sample was dried at room temperature and an ethanol-water (1:1) extraction was performed using maceration method by soaking in the solvent mixture. The extract was collected after removing the solvent and vacuum evaporation in a rotary evaporator.

**Determination of total flavonoids**

The aluminum chloride method was used for the determination of the total flavonoid content of the extracts. Aliquots of extract solutions were taken and made up the volume 3ml with methanol. Then 0.1ml AlCl₃ (10%), 0.1ml Na-K tartarate and 2.8 ml distilled water were added sequentially. The solution mixture was vigorously shaken. Absorbance at 415 nm was recorded after 30 minutes of incubation. A standard calibration plot was generated at 415 nm using known concentrations of quercetin. The concentrations of flavonoid in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent /g of sample.

**Determination of total phenolics**

The total phenolic contents of extracts of *H. pubescens* were determined according to the method described by Malik and Singh. Aliquots of the extracts were taken in a 10 ml glass tube and made up to a volume of 3 ml with distilled water. Then 0.5 ml folinciocalteau reagent (1:1 with water) and 2 ml Na₂CO₃ (20%) were added sequentially in each tube. The tubes with solution were warmed for 1 minute, then cooled. A blue color was developed in each tube because the phenols undergo a complex redox reaction with phosphomolibdic acid in folinciocalteau reagent in alkaline medium which resulted in a blue colored complex. Absorbance was measured at 760 nm. A standard calibration plot was generated at 760 nm using known concentrations of catechol. The concentrations of phenols in the test samples were calculated from the calibration plot and expressed as mg catechol equivalent of phenol/g of sample.

**DPPH radical scavenging activity**

Antioxidants react with 1, 1- diphenyl -2-picryl-hydrazyl (DPPH) radical and convert it to 1, 1-diphenyl -2-picryl hydrazine. The degree of change in colour from purple to yellow can be used as a measure of the scavenging potential of antioxidant extracts. Aliquots of extract solutions were taken and made up the volume to 3ml with methanol. 0.15ml of freshly prepared DPPH
solution was added, stirred and left to stand at room temperature for 30 minutes in dark. The control contains only DPPH solution in methanol instead of sample while methanol served as the blank (negative control). Absorbance was noted at 517 nm by using UV-Vis spectrophotometer. The capacity of scavenging free radicals was calculated as follows:

\[
\text{Scavenging activity (\%) = } \left( \frac{\text{Control abs.- sample abs.}}{\text{Control abs.}} \right) \times 100
\]

IC\(_{50}\) value was calculated from the plotted graph of scavenging activity against the concentrations of the samples. IC\(_{50}\) is defined as the total antioxidant necessary to decrease the initial DPPH radical by 50%. Triplicate measurements were carried out and IC\(_{50}\) was calculated for all the extracts based on the percentage of DPPH radicals scavenged. Ascorbic acid was used as the reference compound (positive control) with concentrations 20 to 500 μg/ml.

**Metal chelating activity**\(^{10}\)

The chelation of ferrous ions by *H. pubescens* extract was estimated by the method of Dinis et al. Briefly, 50μl of 2 mM FeCl\(_2\) was added to 1 ml of different concentrations of the extract (0.2, 0.4, 0.8, 1.6, and 3.2mg ml\(^{-1}\)). The reaction was initiated by the addition of 0.2 ml of 5 mMferrozine solution. The mixture was vigorously shaken and left to stand at room temperature for 10 min. The absorbance of the solution was thereafter measured at 562 nm. The percentage inhibition of ferrozine–Fe\(^{2+}\) complex formation was calculated as \[
\left( \frac{A_0 - A_s}{A_s} \right) \times 100
\]
where \(A_0\) is the absorbance of the control and \(A_s\) is the absorbance of the extract/standard. Na\(_2\)EDTA was used as positive control.

**Assay of nitric oxide-scavenging activity**\(^{11}\)

Sodium nitroprusside (10 mM), in phosphate-buffered saline, was mixed with different concentrations of *H. pubescens* extract dissolved in water and incubated at room temperature for 150 min. Griess reagent (0.5 ml), containing 1% sulfanilamide, 2% H\(_3\)PO\(_4\) and 0.1% N-(1-naphthyl) ethylenediaminedihydrochloride, was added to the mixture after incubation time. The absorbance of the chromophore formed was read at 546 nm. Quercetin and the same mixture of the reaction without extract were employed as positive and negative control.

**Reducing power determination**\(^{12}\)

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action. The reducing power of *H. pubescens* was determined according to the method of Yen and Chen. Different amounts of the extract (0.025-0.4 μg/ml) in water were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium
ferricyanide [K₃Fe(CN)₆] (2.5 ml, 1%). The mixture was incubated at 50 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and FeCl₃ (0.5 ml, 0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. Vitamin C was used as positive control.

Antioxidant Activity by ferric thiocyanate (FTC) method

Two ml of 0.4 mg ml⁻¹ H. pubescens extract was mixed with 2.88 ml of linoleic acid (2.51%, v/v in 4 ml of 99.5% (w/v) ethanol), 0.05 M phosphate buffer pH 7.0 (8 ml), and distilled water (3.9 ml) and incubated at 40 °C for 96 h. To 0.1 ml of this solution, 9.7 ml of 75% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate were then added. Precisely 3 min after the addition of 0.1 ml of 20 mM ferrous chloride in 3.5%(v/v) hydrochloric acid to the reaction mixture, the absorbance at 500 nm of the resulting red solution was measured, and it was recorded again every 24 h until the day when the absorbance of the control reached the maximum value. The percent inhibition of linoleic acid peroxidation was calculated as:

\[ \text{% inhibition} = 100 - \left( \frac{\text{absorbance increase of the sample}}{\text{absorbance increase of the control}} \right) \times 100 \]

All tests were run in duplicate and analyses of all samples were run in triplicate and averaged. Vitamin C and BHA were used as positive control.

Statistical analysis

All values are expressed as mean ± S.E. Statistical analyses were performed by Student's t-test. The values of P lower than 0.05 were considered statistically significant.

RESULT AND DISCUSSION

Total flavonoid and total phenol content of the extracts

It has been recognized that flavonoids show antioxidant activity and their effects on human nutrition and health are considerable. The mechanisms of the actions of flavonoids are through scavenging or chelating processes. Phenolic compounds are a class of antioxidant agents acting as free radical terminators. The flavonoid content of the extract in terms of quercetin equivalent was 37.22 ± 1.34 mgg⁻¹. The total phenol content was measured by FolinCiocalteu reagent in terms of gallic acid equivalent. The total obtained phenol was 78.45 ± 2.78 mgg⁻¹. The amount of polyphenolic compounds in H. pubescens different extracts is dependent on its origin. In
comparison with previous reports, polyphenolic content of *H. pubescens* extract was observable in our study. The compounds, such as flavonoids, which contain hydroxyl groups, are responsible for the radical scavenging effect in the plants. According to our study, the contents of these phytochemicals in *H. pubescens* extract can explain its antioxidant activity.

![Calibration Curve for Total Flavonoid content](image1.png)

![Calibration Curve for Total Polyphenols](image2.png)

**Antioxidant activity**

The stable free radical DPPH method is an easy, rapid, and sensitive way to survey the antioxidant activity of a specific compound or plant extracts. The capacity of *H. pubescens* extract to scavenge DPPH was measured. The amount of reduced DPPH could be quantified by measuring the decrease in absorbance at 517 nm. *H. pubescens* extract reduced DPPH radicals in a dose dependent manner. IC$_{50}$ of the standard compounds, BHA, Vitamin C, and quercetin were...
0.05 μg ml, 0.005 μg ml⁻¹ and 0.005 μg ml⁻¹ respectively. The extract at 800 mg ml⁻¹ scavenged about 91% of DPPH radicals and had an IC₅₀ value of 590 mg ml⁻¹. So the extract showed less potency than the controls in this study. The DPPH scavenging ability of the extract may be attributed to its hydrogen donating ability.

**Metal chelating activity**

Ferrozine can quantitatively form complexes with Fe²⁺. However, in the presence of chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. Measurement of color reduction, therefore, allows the estimation of the chelating activity of the coexisting chelator¹⁶. The transition metal ion Fe²⁺ possesses the ability to move single electrons by virtue of which it can allow the formation and propagation of many radical reactions, even starting with relatively nonreactive radicals. The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions¹⁷. *H. pubescens* extract interferes with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity, and captures ferrous ion before ferrozine. IC₅₀ of the extract for chelating activity was 2 mg ml⁻¹, which is lower than the positive standard EDTA (IC₅₀ = 17.4 mg ml⁻¹).

**Assay of nitric oxide-scavenging activity**

The procedure is based on the principle that sodium nitroprusside in aqueous solution at physiological pH spontaneously generates nitric oxide, which interacts with oxygen to produce nitrite ions that can be estimated using Griess reagent¹⁸. *H. pubescens* extract as a scavenger of nitric oxide competed with oxygen, leading to reduced production of nitrite ions. The IC₅₀ of the extract and quercetin were 552 and 17 mg ml⁻¹. *H. pubescens* extract showed a weaker potency than quercetin in this study. In addition to reactive oxygen species, nitric oxide is also a factor involved in inflammation, cancer, and other pathological conditions. Natural extracts may have the property to counteract the effect of NO formation and, in turn, may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. Furthermore, the scavenging activity may also help to arrest the chain of reactions initiated by excess generation of NO that are detrimental to human health.

**Reducing power**

Fe (III) reduction is often used as an indicator of electron-donating activity, which is an important mechanism of phenolic antioxidant action. The reducing power was determined
According to the method of Yen and Chen. In the reducing power assay, the presence of antioxidants in the samples would result in the reducing of Fe$^{3+}$ to Fe$^{2+}$ by donating an electron. Amount of Fe$^{2+}$ complex can then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in the reductive ability. The extract exhibited a good reducing power at 0.8 and 1.6 mg ml$^{-1}$ that was comparable with Vitamin C (P > 0.05). Because the reductive ability of the extract was significantly comparable to Vitamin C, it was evident that the extract showed reductive potential and could serve as electron donor, terminating the radical chain.

**FTC Method**

Membrane lipids are rich in unsaturated fatty acids that are most susceptible to oxidative processes. Especially, linoleic and arachidonic acid are targets of lipid peroxidation. The inhibition of lipid peroxidation by antioxidants may be due to their free radical-scavenging activities. *H. pubescens* extract displayed a comparable antioxidant activity to the reference standards.

**CONCLUSION**

Using plants as a good source of antioxidants have been examined by many researchers. In our previous studies, were found several plants showing potent antioxidant activity. In the present study, *H. pubescens* extract bears comparable antioxidant activity to the standard compounds. Its constituents scavenge free radicals, chelate the catalytic metal ions, and may exert a protective effect against oxidative damage induced to cellular macromolecules. Free radicals are often generated as byproducts of biological reactions or from exogenous factors. The involvements of free radicals in the pathogenesis of a large number of diseases are well documented. A potent scavenger of free radicals may serve as a possible preventative intervention for the diseases. The preliminary chemical examination of alcoholic-water extract has shown the presence of phenols and flavonoids, which may be responsible of the antioxidant and lipid peroxidation inhibitory activities. The high scavenging property of *H. pubescens* may be due to hydroxyl groups existing in the phenolic compounds’ chemical structure that can provide the necessary component as a radical scavenger. Further studies on the isolation of these compounds are in progress. *H. pubescens* extract activity may be related to the high amount of flavonoid and phenolic compounds leading to an antioxidant activity in the extract.
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REFERENCES


