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Evaluation of *Abelmoschus Esculentus* Seed for Antioxidant and Phytochemical Analysis Using *In vitro* Assays

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Abstract

The aim of this study was to assess the *in vitro* potential of methanolic seed extract of *Abelmoschus esculentus* as a natural antioxidant. The DPPH activity of the Ethyl acetate soluble fraction (10, 20, 40, 80, and 160 μg/mL) was increased in a dose dependent manner, which was found in the range of 18.97-90.47% as compared to ascorbic acid 26.44-93.71%. The IC_{50} values of Ethyl acetate soluble fraction (EAES) and ascorbicacid in DPPH radical scavenging assay were obtained to be 28.12 and 18.43 μg/mL, respectively. Measurement of polyphenol content of the EAES of *A. esculentus* seed was achieved using Folin-Ciocalteau reagent containing 53.80 mg/g of total phenolic content, which was found signicantly higher when compared to reference standard gallic acid. Similarly total flavonoids and proabthocyanidis of EAES and chloroform soluble fraction (CAES) were found significantly 147 mg/g and 14.24 mg/g respectively when both compared to reference standard quercetin. EAES exhibited high significant lipid peroxidation inhibition effects in a dose-dependent manner, with IC_{50} values of 38.08 μg/mL, whereas, standard quercetin, with IC_{50} value of 36.67 μg/mL. All extract/fractions showed dose dependent reducing power ability and these differences were statistically significant ($p<0.001$). The results obtained in this study clearly indicate that *A. esculentus* seed has a signicant potential to use as a natural antioxidant agent.

Key words: *A. Esculentus* Seed, Antioxidant, DPPH, Lipid Peroxidation, Reducing Power

1. Introduction

Significant evidence shows that more than 100 diseases including atherosclerosis, cancer, diabetes, acquired immunodeficiency syndrome (AIDS) and heart diseases affecting human beings are caused or provoked by accumulation of free radicals or reactive oxygen species and the associated lipid peroxidation in the body[1-3]. It is the most important non-infective epidemic to hit the world in the present millennium. The number of people suffering from diabetes worldwide is rising at a frightening rate and predicated that about 366 million people are likely to be diabetic by the year 2030[4]. Hyperglycemia can be handled initially with oral synthetic agents and insulin therapy. However, these synthetic agents produce some serious side effect and the toxicity of oral anti-diabetic agents differs widely in clinical manifestations, severity, and treatment[5]. In the natural system of medicine many plants have been claimed to be useful for the treatment of many diseases.

Plants produce wide array of bioactive principles and constitute a rich source of medicines. In many developing countries, traditional medicine is one of the primary health care systems[6,7]. Large scale evaluation of the local flora exploited in traditional medicine for various biological activities is therefore necessary. Isolation and characterization of the bioactive principles ultimately leading to new drug development. In view of this our attention has been focused particularly *Abelmoschus esculentus* belongs to the family "Malvaceae" commonly known as ladies finger and in several other vernacular names is cultivated as an important vegetable crop in tropical, subtropical and warm temperate regions around the world. Based on these reports our studies have been considered to observe whether the methanolic extract and organic soluble fractions of *Abelmoschus esculentus* Seed exerts *in vitro* antioxidant activity.

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2. Experimental Section

2.1. Plant Materials and Extraction

The fresh seeds of *Abelmoschus esculentus* were collected from Sunder bans, Bagerhat in the month of September 2009 and identified by DR. M.A. Razzaque Shah PhD, Tissue Culture Specialist, BRAC Plant Biotechnology Laboratory, Bangladesh. The dried and coarsely powdered seed (400 g) were extracted with methanol at room temperature for 72 h. The filtrate was evaporated to dryness under reduced pressure (45°C) to afford the crude extract (yield ca. 6%) used in pharmacological screening.

2.2. Drugs and Chemicals

Ammonium molybdate, Folin-chiocaltu phenol reagent, ascorbic acid were purchased from E. Merck (Germany). 1,1-diphenyl-2-picryl-hydrazyl (DPPH), ascorbic acid, quercetin, and potassium ferric cyanide were purchased from Sigma Chemical Company (St. Louis, MO, USA). Normal saline solution was purchased from Beximco Infusion Ltd., Bangladesh. All chemicals used were of analytical reagent grade.

2.3. *In vitro* Antioxidant Activity

2.3.1. Determination of Total Phenolics

The total phenolic content of methanolic extract and several organic fractions were determined using Folin–Ciocalteu reagent[8]. Organic fractions (100 μL) were mixed with the Folin-Ciocalteu reagent (500 μL) and 20% sodium carbonate (1.5 mL). The mixture was shaken thoroughly and made up to 10 mL with distilled water. The mixture was allowed to stand for 2 h. Then the absorbance at 765 nm was determined. These data were used to estimate the phenolic contents using a standard curve obtained from various concentration of gallic acid.

2.3.2. Determination of Total Flavonoids

The flavonoids content was determined by aluminium chloride colorimetric method[9]. Quercetin was used to make the calibration curve. The different concentration of extract (0.5 mL) were separately mixed with 95% ethanol (1.5 mL), 10% alumininum chloride (0.1 mL), 1 M potassium acetate (0.1 mL) and distilled water (2.8 mL). After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm. The amount of 10% aluminum chloride was substituted by the same amount of distilled water in blank. All the determinations were carried out in duplicates. These data were used to estimate the flavonoid contents using a standard curve obtained from various concentration of quercetin.

2.3.3. Determination of Total Proanthocyanidins

Determination of proanthocyanidins was based on the Sun et al. procedure[10]. Exactly 0.5 mL of 0.1 mg/mL of extract solution was mixed with 3 mL of 4 % vanillin-methanol solution and 1.5 mL hydrochloric acid. The mixture was allowed to stand for 15 min. The absorbance was read at 500 nm. Total proanthocyanidins contents were calculated as quercetin acid equivalents (mg/g).

2.3.4. Determination of Total Antioxidant Capacity

The antioxidant activity of the extract/fractions were evaluated by the phosphomolybdenum method according to the procedure of Prieto *et al.*[11]. The assay is based on the reduction of Mo (VI)-Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. Extract (0.3 mL) was combined with 3 mL of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (Shimadzu, UV-150-02) against blank after cooling to room temperature. Methanol (0.3 mL) is used as the blank experiment. The antioxidant activity is expressed as the number of equivalents of ascorbic acid using the following formula,

$$C = (c \times V)/\mathrm{m}$$

Where C is total antioxidant activity, mg/g plant extract, in Ascorbic acid; c is the concentration of ascorbic acid established from the calibration curve, mg/ml; V is the volume of extract, ml; m is the weight of pure plant extract, g.

2.4. Free Radical Scavenging Activity Measured by 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH)

The free radical scavenging activity of extract/fractions, based on the scavenging activity of the stable

1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was determined by the method described by Braca *et al*.[12]. Extract/fractions (0.1 mL) was added to 3 mL of a 0.004% MeOH solution of DPPH. Absorbance at 517 nm was determined after 30 min, and the percentage of inhibition (% I) activity was calculated from following equation,

$$\% I = [(A_0 - A_1)/A_0] \times 100$$

Where A_0 is the absorbance of the control, and A_1 is the absorbance of the extract/standard. IC_{50} value was calculated from the equation of line obtained by plotting a graph of concentration (μg/mL) versus % inhibition.

2.5. Reducing Power Activity

The reducing power of extract/fractions were determined according to the method previously described by Yu L *et al*. [13]. Extract at different concentrations in 1 mL of 10% DMSO were mixed with 2.5 mL of phosphate buffer (0.2 M, pH=6.6) and 2.5 mL potassium ferricyanide [$K_3Fe(CN)_6$] (1%), and then the mixture was incubated at 50 °C for 30 min. Afterwards, 2.5 mL of trichloroacetic acid (10%) was added to the mixture, which was then centrifuged at 3000 rpm for 10 min. Finally, 2.5 mL of upper layer solution was mixed with 2.5 mL distilled water and 0.5 mL $FeCl_3$ (0.1%), and the absorbance was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power.

2.6. Lipid Peroxidation Inhibition Assay

The lipid peroxidation inhibition assay (LPI) was determined according to the method described by Liu *et al*.[14] with a slight modification. Excised rat liver was homogenized in buffer and then centrifuged to obtain liposome. 0.5 mL of supernatant, 100 μL 10 mM $FeSO_4$, 100 μL 0.1 mM AA and 0.3 mL of extractives or standard at different concentration were mixed to make the final volume 1 mL. The reaction mixture was incubated at 37°C for 20 minutes. 1 mL of (28%) TCA and 1.5 mL of (1%) TBA was added immediately after heating. Finally, the reaction mixture was again heated at 100°C for 15 min. and cool at RT. After cooling, the absorbance was taken at 532 nm. Percentage inhibition of lipid peroxidation (% LPI) was calculated by the following equation,

$$\% \text{ LPI} = [(A_o - A_1)/A_o] \times 100$$

Where A_o in the absorbance of the control, and A_1 is the absorbance of the extractives/standard. Then % of inhibition was plotted against concentration and from the graph IC_{50} was calculated.

2.7. Statistical Analysis

All data were expressed as mean±S.E.M. One-way ANOVA followed by Dunnett's multiple comparison tests was used to analyze the data obtained from *in vivo* experiments. All statistical analyses were performed with Prism 4.0 (GraphPad software Inc., San Diego, CA). $P<0.05$ was considered to be significant.

3. Results and Discussion

3.1. Total Phenol, Flavonoid and Proanthocyanidin Content

The results of total phenol, total flavonoids and total proanthocyanidins contents of methanolic extract of *Abelmoscus esculentus* seed and its organic soluble fractions are presented in Table 1. The results show that EAES (67.33 mg/g) had highest content of total phenolic contents then followed by MAES > CAES > WAES. The flavonoids content of EAES (147.40 mg/g) is the highest and then followed by MAES > WAES > CAES. The CAES (14.24 mg/g) had highest content of proanthocyanidins compared to MAES > EAES > WAES.

3.2. Total Antioxidant Capacity

Total antioxidant capacity is given in Fig. 1. EAES extract showed the highest total antioxidant capacity is expressed as the number of equivalent of ascorbic acid

Table 1. Polyphenol contents of the fractions (mg/g) of *Abelmoschus esculentus* seed

Polyphenols	MAES	EAES	CAES	WAES
Total phenolics	33.96	53.80	67.33	9.33
Total Flavonoids	71.40	147.40	18.40	44.20
Proabthocyanidis	7.38	3.65	14.24	1.44

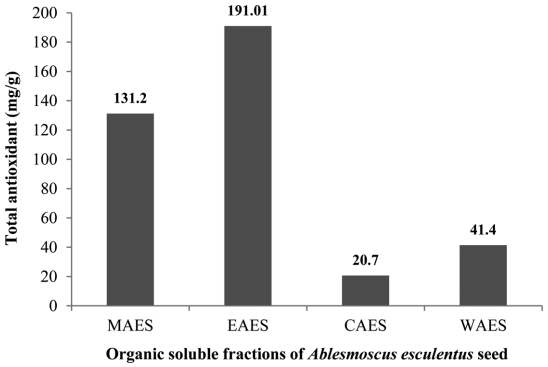

Fig. 1. Total anioxidant (mg/g) of different organic soluble fractions of *Ablesmoscus esculentus* seed.

and was found to be 191.01 mg/g then followed by MAES > WAES > CAES.

3.3. DPPH Radical Scavenging Activity

Fig. 2 shows that the dose-response curve of DPPH radical scavenging activity of the different extracts of *Abelmoscus esculentus* seed compared with ACA. All the fractions showed dose dependent activity demonstrated that extract or fractions have proton-donor activity. EAES showed the highest DPPH scavenging activity with the IC_{50} value of 28.12 μg/mL, followed by MAES and WAES with the IC_{50} value of 61.61 and 93.01 μg/mL, respectively. CAES had no activity within the experimental concentration range whereas standard ascorbic acid showed IC_{50} value of 18.43 μg/mL.

The effect of antioxidants on DPPH is thought to be due to their hydrogen donating ability[15]. Radical scavenging activities are very important to prevent the deleterious role of free radical in different diseases including cancer. Our results revealed that the methanolic extract of *Abelmoscus esculentus* seed and its organic soluble fractions had the similar free radical scavenging activity when compared with standard ACA. Based on the data obtained from this study, DPPH radical scavenging activity of *Abelmoscus esculentus* seed (IC_{50} 28.12 μg/mL) was similar to the standard ascorbic acid (IC_{50} 18.43 μg/mL) (Fig. 2.).

3.4. Lipid Peroxidation Inhibition Activity

EAES exhibited high significant Lipid peroxidation inhibition effects in a dose-dependent manner, with IC_{50} values of 38.08 μg/mL, followed by MAES (56.59 μg/mL), WAES (91.66 μg/mL), and CAES (134.7 μg/mL) whereas, quercetin with IC_{50} value of 36.67 μg/mL. (Fig. 3).

3.5. Reducing Power Ability

For the measurement of the reductive ability, we investigated the Fe^{3+} to Fe^{2+} transformation in the presence of extract and organic fractions. Like the antioxidant activity, the reducing power of EAES increased with increasing concentration of the sample. Fig. 4 shows the reductive capabilities of the EAES compared with ascorbic acid and gallic acid. All extract/fractions showed dose dependent activity and these differences were statistically significant ($p<0.001$).

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity[16]. The reducing properties are generally associated with the presence of reductones which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom[17]. Our data on the reducing power of the tested extracts recommended that

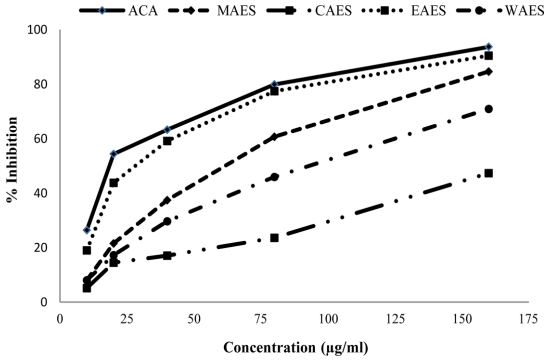

Fig. 2. Comparative % inhibition of DPPH showed by standard antioxidant (ascorbic acid) and different fractions.

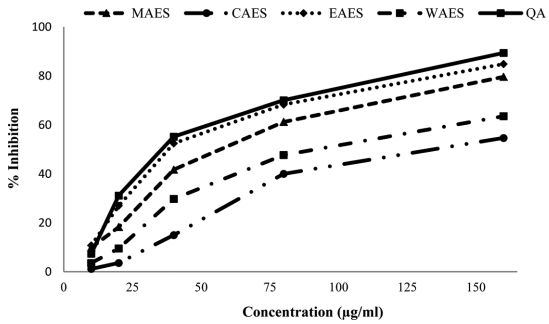

Fig. 3. Comparative % inhibition of Lipid peroxidation by standard peroxidant (Quercetin) and different fractions.

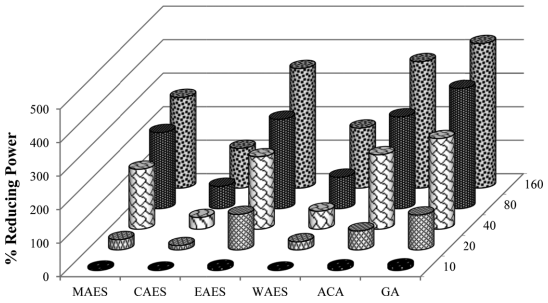

Fig. 4. Comparative % of reducing power with standard Ascorbic acid and Gallic acid as well as different fractions.

it is likely to throw in significantly towards the observed antioxidant effect. Phytochemicals especially polyphenols have received increasing attention because of interesting new discoveries considering their biological activities[18]. It is found that in current study, a number of phytochemical are present in the extract of this species and they constitute a major group of compounds that acts as primary antioxidants with high redox potentials and singlet oxygen quenchers [19].

4. Conclusions

As a conclusion, it could be speculated that the observed anti-oxidative activity of EAES might be connected to the presence of polyphenol as active constituents. The present investigation has also opened an opportunity for further research especially with reference to the development of potent formulation for oxidative stress from EAES. However, these effects need to be confirmed by employing different *in vivo* models and clinical trials for their effective utilization as therapeutic agents.

Abbreviation

DPPH = 2, 2-Diphenyl-1-Picrylhydrazyl
ACA = Ascorbic Acid
MAES = Methanol Soluble Fraction
QA = Quercetin
CAES = Chloroform Soluble Fraction
WAES = Water Soluble Fraction
GA = Gallic Acid
EAES = Ethyl Acetate Soluble Fraction

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