In vitro antioxidant activity of banana (*Musa* spp. ABB cv. Pisang Awak)

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The methanolic extract of *Musa* ABB cv Pisang Awak was investigated for the polyphenolic contents and antioxidant activity. The total phenol and flavonoid contents of the fruit extract were found to be 120 mg gallic acid equivalents (GAE) and 440 mg quercetin equivalents (QE)/100 g of sample dry weight, respectively. The antioxidant activity of the Pisang Awak methanol extract (PAME) (20-500 µg/ml) was determined using 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, reducing capacity, 2,2'-azinobis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS) radical cation decolourization and hydroxyl radical scavenging capacity (OH•). The EC50 values of DPPH, ABTS and OH• activities of the PAME and butylated hydroxy toluene (BHT) were found to be 65 and 9 µg/ml, 29 and 6 µg/ml, 36 and 42 µg/ml respectively. The reducing capacity increased with increasing concentration (31.5-1000 mg/ml) of the fruit extract and the activity was comparable with the standard BHT. The high performance thin layer chromatography (HPTLC) analysis of the extract revealed the presence of polyphenols. The strong and positive correlations were obtained between total phenol/flavonoid contents (R2 = 0.693-1.0) and free radical scavenging ability was attributed to the polyphenols as the major antioxidants.

**Keywords:** Banana, Pisang Awak, Antioxidant activity, Polyphenols, Flavonoids, Reducing power, DPPH, Hydroxyl radical, ABTS, high performance thin layer chromatography.

The consumption of fruits and vegetables is thought to be associated with a reduced risk of many diseases including cancer, atherosclerosis and neurodegenerative diseases, which are related to elevated levels of oxidative stress1,2. Antioxidant compounds can decrease oxidative stress and minimize the incidence of these diseases3,4. The mechanism of the action of these antioxidant compounds include suppression of reactive oxygen species formation either by inhibition of the enzymes or by chelation of trace elements involved in free radical production, scavenging of reactive species and up-regulating or protecting antioxidant defense5.

Banana (*Musa*) is one such fruit yielding tropical plant that may protect itself from the oxidative stress caused by strong sunshine and high temperature by producing large amounts of antioxidants6. Banana fruits contain various antioxidant components, such as vitamin C, vitamin E, β-carotene and polyphenolics7. Antioxidant capacity of a fruit is mainly due to component, such as flavonoids. Further, the antioxidant capacity of banana may also be attributed to the presence of gallicatechin and dopamine10,11. These compounds play an important role in protecting neuronal cells from oxidative stress induced neurotoxicity12.

In the present study, antioxidant potential of *Musa* sp. ABB cv. Pisang Awak, which is widely cultivated and consumed in central province of Tamil Nadu and locally known as Karpooravally has been evaluated using 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity, reducing capacity (RC), 2,2’-azinobis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS) radical cation decolourization and hydroxyl radical (OH•) scavenging capacity. The polyphenolic compounds in the extract have also been determined by high performance thin layer chromatography (HPTLC).

**Materials and Methods**

**Chemicals**

1,1-Diphenyl-2-picryl hydrazyl (DPPH), 2,2’-azinobis-3-ethylbenzothiozoline-6-sulfonic acid (ABTS), quercetin and butylated hydroxy toluene (BHT) were purchased from Merck (Mumbai). Potassium ferricyanide, Folin-Ciocalteu reagent, methanol, gallic acid, quercetin, trichloroacetic acid (TCA), ferric chloride, sodium carbonate, potassium dihydrogen phosphate, dipotassium hydrogen phosphate, aluminium...
chloride and potassium persulfate were obtained from SD fine Chemicals, India. All chemicals used including the solvents were of analytical grade.

**Fruit collection and preparation of extract**

The fruits of Pisang Awak were collected from banana cultivating farms from Pollachi in Tamil Nadu, India. The plants were identified and authenticated by Botanical Survey of India (BSI), Coimbatore. The fruit material was freeze-dried in the lyophilizer (Alpha 1-2 LD) and powdered. The powder (50 g) was extracted with 250 ml methanol using an orbital shaker for 42 h, centrifuged at 9000 rpm thrice and supernatants were combined. The combined supernatant was filtered over Whatman No.1 filter paper and then evaporated to dryness by rotary flash evaporator (Buchi type). Different concentrations of extracts were prepared from resultant Pisang Awak methanol extract (PAME) to determine in vitro antioxidant activities.

**Determination of total phenolic content**

Total phenolic content (TPC) were determined by Folin-Ciocalteu method\(^{13}\). Briefly, an aliquot of the sample extract (0.1 ml) was mixed with distilled water (3 ml) and 0.5 ml of Folin-Ciocaltelu reagent was added. After 3 min, 2 ml of 20% sodium carbonate was added and mixed thoroughly. The tubes were incubated in a boiling water bath for exactly 1 min, then cooled and the absorbance was measured at 650 nm using spectrophotometer (Elico Scanning mini spec SL 177, India) against the reagent blank. TPC was expressed as mg gallic acid equivalent (GAE)/100 g sample dry weight.

**Determination of total flavonoid content**

Total flavonoid content (TFC) was determined using a standard method of Ordon\textit{Ez et al.}\(^{14}\). Briefly, an aliquot of 0.5 ml of 2% AlCl\(_3\) was added to 0.5 ml of sample solution. After 1 h at room temperature, the absorbance was measured at 420 nm at the final concentration of 0.1 mg/ml. TFC was calculated as mg quercetin equivalent (QE)/100 g sample dry weight.

**DPPH radical scavenging assay**

The radical scavenging activity of PAME was measured in terms of hydrogen donating or radical-scavenging ability using the method of Singh \textit{et al.}\(^{15}\). Different concentrations (25-500 µg/ml) of samples and BHT were taken. To about 5 ml of 0.1 mM methanolic solution DPPH was added and shaken vigorously. After incubation at 27°C for 20 min, the absorbance was measured at 517 nm. The radical inhibition percentage was calculated using the following formula: DPPH radical scavenging activity (%) = (Absorbance \text{control} - Absorbance \text{sample})/Absorbance \text{control}) \times 100. BHT was used as reference standard.

**Ferric reducing power (FRP)**

The reducing capacity of PAME was determined according to the method of Oyaizu\(^{16}\). Various concentrations of the extract (31.5-1000 µg/ml) were added to 2.5 ml of (0.2 M) sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide \([K\text{}_3\text{Fe}(CN)\text{6}]\) solution. The contents were vortexed well and incubated at 50°C for 20 min. After incubation, 2.5 ml of 10% TCA was added to all the tubes and centrifugation was carried out at 3000 g for 10 min. Thereafter, to 5 ml of the supernatant, 5 ml of deionized water was added. To this, 1 ml of 1% ferric chloride was added to each test tube and incubated at 35°C for 10 min. The formation of Perl’s Prussian colour was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. BHT was used as reference standard.

**ABTS\(^{+}\) radical scavenging assay**

ABTS\(^{+}\) radical scavenging assay of PAME was determined based on the method described previously\(^{17}\). The stock solutions included 7 mM ABTS solution and 2.4 mM potassium persulfate solution. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 h at room temperature in the dark. The solution was then diluted by mixing 1 ml ABTS solution with 60 ml methanol to obtain an absorbance of 0.706 ± 0.001 units at 734 nm using the spectrophotometer. The fruit extract and BHT of various concentrations (20-100 µg/ml) were allowed to react with 1 ml of the ABTS solution and the absorbance was read at 734 nm after 7 min. BHT was used as reference standard. The ABTS\(^{+}\) scavenging capacity was calculated as ABTS\(^{+}\) scavenging activity (%) = [(Absorbance \text{control} - Absorbance \text{sample})]/(Absorbance \text{control})] \times 100.

**Hydroxyl (OH\(^{-}\)) radical scavenging activity**

The OH\(^{-}\) scavenging activity of PAME was measured according to the method of Klein \textit{et al.}\(^{18}\). Various concentrations (20-100 µg) of extract were added with 1.0 ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), and 1.0 ml of DMSO
(0.85% v/v in 0.1 M phosphate buffer, pH 7.4). The reaction was initiated by adding 0.5 ml of ascorbic acid (0.22%) and incubated at 80-90°C for 15 min in water bath. After incubation, the reaction was terminated by the addition of 1.0 ml of ice-cold TCA (17.5% w/v). To about 3 ml of Nash reagent (75 g of ammonium acetate, 3 ml of glacial acetic acid, and 2 ml of acetyl acetone were mixed and raised to 1 L with distilled water) was added and left at room temperature for 15 min. The intensity of the color formed was measured at 412 nm against the reagent blank. The OH- formed was measured at 412 nm against the reagent temperature for 15 min. The intensity of the color formed was measured at 412 nm against the reagent blank. The OH- scavenging activity (%) was calculated as 1 - (Difference in absorbance sample/difference in absorbance blank) × 100.

HPTLC analysis
A densitometric HPTLC analysis of PAME was performed for the development of characteristic fingerprinting profile. The PAME extract was dissolved with HPLC grade methanol 100 mg/0.5 ml. The solution was centrifuged at 3000 rpm for 5 min and used for HPTLC analysis. The samples (2 µl) were loaded as 7 mm band length in the 10 × 10 Silica gel 60F 254 TLC plate using Hamilton syringe and CAMAG LINOMAT 5 instrument. The samples loaded plate was kept in TLC twin trough developing chamber (after saturation with solvent vapour) with toluene-acetone-formic acid (4.5:4.5:1 v/v/v) as mobile phase. Finally, the plate was fixed in scanner stage and scanning was done at 254 nm. The peak table, display and densitogram were identified.

Statistical analysis
The results were expressed as mean (n = 3) ± standard deviation (SD). The percentage inhibition vs. concentration was plotted and the concentration required for 50% inhibition of radicals was expressed as EC50.

Results and Discussion
Total phenol and flavonoid contents in the extract
Total phenol and flavonoid contents of the PAME were found to be 120 ± 5.9 mg GAE and 440 ± 3.2 mg QE/100 g sample dry weight. The content of phenolics in Pisang Awak was higher than that of Musa sapientum, pisang mas cultivar (51 ± 7.0)20, M. balbisiana (Harton plane) (115.01 ± 0.31) and less than that of M. cavendish (122.02 ± 0.19) and M. manzano (153.17 ± 0.21)20. Further, TPC in banana peel of M. acuminata Colla (AAA group) ranges from 0.90 to 3.0 g/100 g sample dry weight10,11,22. However, TPC of Pisang Awak was little higher than that of bananas consumed in American diet23.

Further, it has been reported PC12 cells when pretreated with banana fruit phenolics block oxidative stress-induced cytotoxicity in a dose-dependent manner12. Polyphenolic compounds contribute significantly to the total antioxidant capacity of the fruits24. Presence of the antioxidant activity of flavonoids from banana (M. paradisiaca) has been studied in rats fed normal as well as high fat diet25. A monomeric flavonoid leucocyanidin and the major component present in unripe plantain banana (M. sapientum L. var. paradisiaca) exert anti-ulcer activity26. Further, flavonoid-rich fraction from two varieties of M. paradisiaca is found to responsible for enhanced antioxidant, hypolipidemic and radioprotective action in albino rats27. Flavonoids play some important pharmacological roles against diseases, such as cardiovascular disease, cancer, inflammation and allergy. Epidemiological studies have indicated the relationship between flavonoid intake and reduced risk of certain cancers28.

DPPH radical scavenging assay
DPPH radicals react with suitable reducing agents and then electrons become paired-off and the solution loses colour stoichimetrically with the number of electrons taken up15. Such reactivity has been widely used to test the ability of compounds/plant extracts to act as free radical scavengers. In the present study, reduction of the DPPH radicals was found in concentration-dependent manner (Fig. 1a). The PAME reduced the stable DPPH radical to yellow colored unstable compound, with an EC50 value of 65 ± 1.5 µg/ml which is less than BHT value of 9.0 ± 0.5 µg/ml. The ethanolic extract of green and yellow peel of M. cavendish at the concentration of 0.5-1 mg/ml has shown low DPPH scavenging activity (52 ± 0.2 and 43.7 ± 0.5, respectively) than PAME (68 ± 0.53) at the concentration of 25-500 µg/ml29. On the other hand, methanolic peel extract of M. acuminata Colla AAA cv. Grande Naine and Gruesa has shown the DPPH scavenging activity of 44 ± 8, 42 ± 5, respectively, whereas aqueous extract of peel of banana cv. Grande Naine and Gruesa showed the scavenging activity of 3 ± 0.1 and no activity, respectively. Thus, choosing the appropriate solvent is one of the most important factors in obtaining extracts with a high content of bioactive compounds and antioxidant activity11.
However, BHT displays significant scavenging activity over the PAME. This might be due to the presence of methoxy group which increases the accessibility of radical center of DPPH to BHT.

**Ferric reducing power (FRP)**

The reducing power of the extract was linearly proportional to the concentration of the sample. As can be seen in Fig. 1b, the reducing capacity of the PAME and BHT was found to be 0.654 OD$_{700}$ and 0.709 OD$_{700}$ at 1 mg/ml, respectively. The increased reducing power in the PAME indicated that some components in the extract were electron donors that could react with the free radicals to convert them into more stable products to terminate radical chain reaction. Antioxidants are strong reducing agents and this is principally based on the redox properties of their hydroxyl groups and the structural relationships between different parts of their chemical structure.

**ABTS$^{•+}$ radical scavenging assay**

Proton radical scavenging is an important attribute of antioxidants. ABTS, a protonated radical has characteristic absorbance maxima at 734 nm which decreases with the scavenging of proton radicals. The PAME was effective scavenger of ABTS radical and this activity was comparable to that of BHT (Fig. 1c). The EC$_{50}$ values were 29 ± 0.53 and 6 ± 0.21 µg/ml for the PAME and BHT, respectively. The decolourization of ABTS radical cation reflects the capacity of antioxidants to donate electrons or hydrogen atoms to deactivate these radical species. This may be due to variation in types of phenolic compounds that differ significantly in their reactivity towards ABTS. The methanolic and aqueous extracts of peel of *M. acuminata* Colla AAA cv. Grande Naine and Gruesa have shown the ABTS scavenging activity ranging from 8.3-56% and the activity being lower than the PAME (63%)$^{11}$. In the earlier study, banana has been found to exhibit higher ABTS radical scavenging activity than cherries, grapes, oranges, peach etc. and lower than that of plums, strawberries, apples and lemons$^{36}$.

**OH$^{•}$ radical scavenging activity**

OH$^{•}$ radical is an extremely reactive free radical formed in the biological system and has been implicated as highly damaging species in free radical pathology, capable of damaging almost every molecule found in the living cells and initiator of lipid peroxidation. The percentage radical scavenging activity of the PAME (63 ± 0.25) was examined and compared with BHT (Fig. 1d). The EC$_{50}$ value of the PAME and BHT was found to be 36 and 42 µg/ml, respectively. Similar results have been observed with the scavenging activity of other species of bananas,
such as Manzano banana (66 ± 0.03), Cavendish banana (53.51 ± 0.03) and Harton plane (31.81 ± 0.01)\textsuperscript{21}. The ability of the PAME to quench OH· seems to be directly related to the prevention of the lipid peroxidation and appears to be moderate scavenger of active oxygen species, thus reducing rate of chain reaction\textsuperscript{15}.

**HPTLC analysis of polyphenolic compounds**

The HPTLC fingerprint was used for the identification of polyphenolic compounds present in the extract\textsuperscript{38}. HPTLC profile of the PAME was recorded by the run performed along with the standard polyphenol (catechin). The identity of polyphenols in the PAME was confirmed by the overlaying the UV absorption spectra of the sample with that of the reference standard which showed lambda max at 254 nm. The bands of the PAME revealed the presence of polyphenols (Fig. 2). The standard catechin and the PAME had an \( R_f \) value of 0.64 and 0.67, respectively (Figs 3a and b).

**Correlation analysis**

The linear correlations of different antioxidant assays with contents of fruits were analyzed and represented in Table 1. It was observed that the TPC had a good positive correlation with antioxidant assays, such as DPPH (\( R^2 = 0.955 \)), ABTS (\( R^2 = 0.958 \)), reducing power (\( R^2 = 0.934 \)) and OH· scavenging (\( R^2 = 0.693 \)) activities. The TFC showed a strong correlation with reducing power (\( R^2 = 0.934 \)), DPPH (\( R^2 = 0.955 \)) and ABTS (\( R^2 = 0.966 \)) scavenging activities. Interestingly, the OH· scavenging activity and TFC (\( R^2 = 1.0 \)) exhibited a maximum positive correlation. Therefore, strong and positive correlation between antioxidant activity, flavonoid and phenol contents suggested that antioxidant capacity of the extract was mainly due to the flavonoids and polyphenols\textsuperscript{39,40}. Earlier, similar results have been observed on linear correlation between polyphenolic contents and antioxidant properties\textsuperscript{15}.

Recently, natural foods and food-derived components, such as antioxidative vitamins and phenolic phytochemicals have received a great deal of attention, because they are safe when consumed on regular basis; some of these are known to function as chemoprotective agents against oxidative damage. Consumption of fruits and vegetables has been associated with reduced risk of chronic diseases such as cardiovascular diseases and some types of cancer\textsuperscript{15}.

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Table 1—Correlation coefficient between antioxidant activities, total phenol content (TPC) and total flavonoid content (TFC) of the PAME

DPPH, diphenyl picryl hydrazyl; ABTS, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid); HR, hydroxyl radical scavenging; FRP, ferric reducing power assay.

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Fig. 2—Chromatogram of reference standard catechin (lane 1) and PAME (lane 2) in HPTLC analysis after derivatization

Fig. 3—Peak densitogram display of the standard catechin (a) and PAME (b)
as cardiovascular diseases and cancer. The phytochemicals, especially phenolics in fruits and vegetables are suggested to be the major bioactive compounds for health benefits.

In conclusion, the PAME exhibited considerable free radical scavenging activity which might be attributed due to the presence of phenolics and flavonoids. Consumption of Pisang Awak fruit may supply substantial antioxidants in the form of major flavonoids which may attenuate the oxidative stress, leading to health promoting and disease preventing effects. In addition to consumption of fresh fruits such as apples and oranges, inclusion of this locally available banana cv. Pisang Awak in daily diet may also be beneficial to ameliorate chemopreventive effects in neurodegenerative disease. Therefore, supplementation of banana as a part of balanced diet may enrich the antioxidant source and could be considered for in protection against oxidative stress. However, further research is necessary for the better utilization of this species.

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References