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In-vitro free radical scavenging activities of the leaves of *Malva verticillata* L.

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Abstract

Antioxidants act as major defense against radical-mediated toxicity by protecting against the damages caused by free-radicals. Research on herbal products are increasingly focused on their effects on scavenging of different newly generated free-radical species and associated oxidative stress mediated complications on human health, but there are unexpectedly few studies evaluating the bioactivity of edible leafy vegetables of North Bengal, India. The purpose of the present study was to investigate the *in vitro* antioxidant potential of methanol extract and aqueous decoction of *Malva verticillata* L. [MV] leaves, consumed by local people of North Bengal. Extracts of leaves were analyzed for *in vitro* free radical scavenging capacity, the total phenol and flavonoid content and preliminary phytochemical analysis. The antioxidant property was estimated using reducing power, superoxide radical scavenging activity and DPPH· assays. Methanol extract of leaves were found to be effective in DPPH· and superoxide radical scavenging activity when compared with aqueous decoction of MV. Overall strong correlation between the mean values of total phenol content and IC_{50} values of DPPH and superoxide free radical scavenging capacity was observed. Principle Component Analysis (PCA) indicated that phenolic functional groups and reducing potential of methanol and aqueous extracts were mostly contributed for their antioxidant capacity. The present study revealed that methanol extract of the leaves of MV comprise effective source of natural antioxidants, which might be helpful in preventing the progress of various oxidative stress induced diseases.

Keywords: *Malva verticillata*, Antioxidant, DPPH, reducing power, Photochemical

The role of free radicals in many diseases has been well established. Several biochemical reactions in our body generate reactive oxygen species (ROS) that are capable of damaging crucial biomolecules (Kumaran & Karunakaran, 2006). ROS such as hydroxyl, superoxide and peroxyl radicals are formed in human tissues during metabolic operations which cause extensive oxidative damage that leads to age-related degenerative conditions, cancer, and a wide range of other human diseases (Aruoma, 1999; Reaven & Witztum, 1996). In recent years, one of the areas of therapeutic research which have been fascinated with great deal of attention is antioxidant, especially in the control of degenerative diseases in which oxidative damage has been implicated. Antioxidants play an important role in the protection of the human body against damage by free radicals and always maintain homeostatic balance with pro-oxidants inside cellular compartments (Lollinger, 1981).

Many plants contain substantial amount of antioxidants which can be utilized to scavenge the excess free radicals. The protection offered by different edible plants against oxidative stress in several diseases has been attributed to various antioxidants and vitamins. Potential antioxidant properties of the dietary phenolic compounds and flavonoids present in various fruits and vegetables have recently been recognized in a number of investigations (Mondal *et al.*, 2008; Bhaumik *et al.*, 2008). A great number of aromatic, spicy, medicinal and other plants contain chemical compounds, exhibiting antioxidant properties. Sources of natural antioxidants are primarily plant phenolics that may occur in all parts of plants such as fruits, vegetables, nuts, seeds, leaves, roots and barks (Pratt and Hudson, 1990). Many of these antioxidant compounds possess antiinflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial or antiviral activities to a greater or lesser extent (Halliwell, 1994; Mitscher *et al.*, 1996; Owen *et al.*, 2000; Sala *et al.*, 2002).

Solvent extraction is most frequently used technique for isolation of herbal antioxidant compounds. However, the extractive yields and resulting antioxidant activities of the plant materials are strongly dependent on the nature of extracting solvent, due to the presence of different antioxidant compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent. Polar solvents are frequently employed for the recovery of polyphenols from a plant matrix. The most suitable of these solvents are (hot or cold) aqueous mixtures containing ethanol, methanol, acetone, and ethyl acetate (Peschel *et al.*, 2006).

The plant *Malva verticillata* L. (Malvaceae) is

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commonly known as musk mellow or mellow in Korea and China. The plant has been traditionally used as stomach ailment, anticomplementary, hypoglycemic and postpartum fever in India, China and Korea. (Buragohain 2011; Tomado *et al*., 1992; Jeong & Song 2011). In India, it is widely cultivated throughout temperate Himalayas from Punjab eastwards to Assam. In West Bengal, it is commonly known as 'Lapha' or 'Laffa' and its young tender twigs are cooked as vegetable (Bandyopadhyay and Mukherjee 2009).

A review of the literature did not throw ample light on the antioxidant and phytochemical study of this plant. The present study was therefore carried out to investigate the antioxidant potential of methanol extract and aqueous decoction from the leaves of MV in different *in vitro* models along with qualitative and quantitative analysis of active secondary metabolites for determining their relationship with antioxidants.

Materials and methods

Plant material

The plant material was purchased from the local markets of Shivmandir, West Bengal, India on 8th February 2011. Taxonomic position was authenticated by the Taxonomy and Environmental Biology Laboratory, Department of Botany, University of North Bengal. The material has been deposited in the 'NBU Herbarium' and recorded against the accession number 9622 (Figure 1).

Figure 1: Herbarium specimen of *Malva verticillata* L.

Chemicals

Methanol, (2, 2-diphenyl-1-picryl hydrazyl) DPPH˙, nicotinamide-adenine dinucleotide reduced form (NADH), phenazine methosulphate (PMS), potassium ferricyanide ($K_3[Fe(CN)_6]$), trichloroacetic acid (TCA), ferric chloride ($FeCl_3$), Folin-ciocalteu reagent, sodium carbonate (Na_2CO_3), nitro blue tetrazolium (NBT), sodium nitrite ($NaNO_2$), Griess reagent, aluminium chloride ($AlCl_3$), sodium hydroxide (NaOH), sodium nitroprusside, sodium sulphate (Na_2SO_4), lead acetate, 95% ethanol, 50% hydrochloric acid (HCl) and chloroform were either purchased from Himedia-BDH or Merck, Germany. All chemical and solvents used for experiments were of analytical grade.

Extraction

The leafy parts of MV were first separated from the young twigs. 20 g of leaves were separately crushed using mortar and pestle. After crushing, leaves were extracted separately with hot water and methanol in conical flask for 2 hours. The supernatants of refluxed samples were isolated from the residues by filtering through Whatman No. 1 filter paper. The excess solvent was removed by evaporation at 30° C and final concentration was taken as 500 mg/ml. 1ml of filtrates were dried *in vacuo* through lyophilization and their total extractive values were calculated on dry weight basis by the formula:

$$\text{yield}(\%) = \frac{\text{Wt. of dry extract}}{\text{Wt. taken for extraction}} X100$$

The samples were then kept in freeze for further use.

Extracts of each solvent were evaporated similarly under reduced pressure and final residues were used for assessment of antioxidant activity.

DPPH *free radical scavenging assay*

The free radical scavenging activity of the extracts was determined by Blois (1958). The leaves extracts were measured in terms of hydrogen donating or radical scavenging ability using a stable radical DPPH˙. 0.2ml of plant extracts prepared as various concentrations (5–500 mg/ml) were added to 2 ml of the methanol solution of 0.2mM DPPH, and the mixture was vortexed vigorously. The tubes were then incubated at room temperature for 30 minutes in dark, and the absorbance was taken at 517nm by UV-VIS spectrophotometer (Systronics, 2201). Ascorbic acid was used as reference. The ability to scavenge DPPH radical was calculated by the following equation:

$$\text{DPPH scavenging}(\%) = [(A_{control} - A_{sample})/ A_{control} \times 100]$$

Where; $A_{control}$ is the absorbance of DPPH radical + methanol; A_{sample} is the absorbance of DPPH radical + sample extract /standard

Linear regression analysis was used to calculate IC_{50} values wherever needed. IC_{50} value shows the amount of each extract needed for 50% inhibition of free radicals.

Table 1: Total phenol and flavonol content of MV leaf extracts

Sample	Concen-tration	TPC(mg/g FWT)	TFC(µg/g FWT)
Aqueous decoction	100mg/ml	0.53	25.3
Methanol	100mg/ml	1.73	7.2

TPC=Total phenol content, TFC=Total flavonoid content

Superoxide anions scavenging activity

The superoxide anions generated by PMS and nicotinamide-adenine dinucleotide reduced form (NADH), were detected by the reaction with 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-(3, 3′-dimethoxy-4, 4′-diphenylene) di-tetrazolium chloride (nitro blue tetrazolium-NBT). (Nishikimi, *et al.* 1972). Reaction mixture contained 1 ml samples (different concentration), 1 ml of NBT solution (312µM prepared in phosphate buffer, pH-7.4) and 1ml of NADH solution (936 µM prepared in phosphate buffer, pH-7.4). Finally, the reaction was accelerated by adding 100 µL PMS solution (120 µM prepared in phosphate buffer, pH -7.4) to the mixture. The reaction mixture was incubated at 25° C for 5 min and absorbance at 560 nm was measured against methanol as control. IC_{50} values were calculated by using the same analytical techniques mentioned above.

Reducing power assay

One milliliter of leaf extracts, 2.5 ml sodium phosphate buffer (0.2 M, pH 6.6), and 2.5 ml $K_3[Fe(CN)_6]$ (1% w/v) were incubated at 50°C for 20 minutes. The tube was cooled on ice and 2.5 ml 10% TCA was added. The mixture was centrifuged at 3000 rpm for 10 minutes and the upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and 0.25 ml of $FeCl_3$ (0.1% w/v). Finally, the absorbance was measured at 700nm. Phosphate buffer (pH=6.6) was used as blank solution (Aiyegoro and Okoh, 2009). Increased absorbance of the reaction mixture indicated superior reducing power.

Table 2: Phytochemical analysis of MV leaf extracts

Test	Aqueous decoction	Methanol
Steroid	++	+
Alkaloid	-	-
Cardiac glycoside	++	+
Flavonoid	++	+
Tannin	+	++
Glycosides	+	+
Saponin	+	+
Triterpenoid	-	-

+= Average, + =Minimum activity, - = No activity

Total phenol content

Total phenolic compounds of leaves extracts were determined by Folin-Ciocalteu method. (Folin and Ciocalteu, 1927). For the preparation of the calibration curve, 1 ml aliquot of 0.025, 0.05, 0.075, 0.1, 0.2 and 0.3 mg/ml methanolic gallic acid solution was mixed with 5 ml of Folin-Ciocalteu reagent (10 times diluted) and 4 ml Na_2CO_3 (75 g/L). The tubes were vortexed and allowed to stand for 30 min at 40 °C for colour development .The absorbance at 765 nm was measured after 1 hr. at 20 °C and the calibration curve was drawn. 1 ml plant extracts of various concentration was mixed to the same reagent and the mixture was incubated for one hour in room temperature. After 1 hour the absorbance was measured at 765nm.

Total Flavonoids Content

The total flavonoid content was determined using a spectrophotometric aluminium chloride method (Sultana *et al.*, 2009). 0.5 ml of the sample extracts (0-500 mg L^{-1}) (Quercetin in case of standard) were mixed separately with 4 ml of distilled water in a test tube, followed by the addition of 0.3 ml of 5% $NaNO_2$ solution. After 6 minutes, 0.3 ml of 10% $AlCl_3$ solution was added and the mixture was allowed to stand for 5 minutes before the addition of 2 ml of 1 M NaOH solution. About 2.4 ml of distilled water was finally added and the absorbance was measured immediately at 510 nm.

Phytochemical evaluation of the crude extracts

Test for steroid

0.5ml leaves extracts were evaporated and dissolved in 2ml chloroform. 2ml of conc. H_2SO_4 was introduced carefully by the side wall of the test tube. Formation of red colour ring confirmed the presence of steroid (Kumar *et al.*, 2009).

Test for triterpenoids

0.5 ml of leaves extracts were evaporated and dissolved in 1ml chloroform. 1ml acetic anhydride was then added and chilled. After cooling, conc.H_2SO_4 was added. If

Table 3: Correlation co-efficient matrix for different antioxidants and phytochemical parameters

	DPPHm	SOm	RPm	TPCm	TFCm	DPPHw	SOw	RPw	TPCw
SOm	0.727								
RPm	0.935*	0.885*							
TPCm	0.971**	0.835	0.943*						
TFCm	0.992**	0.713	0.913*	0.978**					
DPPHw	0.656	0.994**	0.829	0.786	0.648				
SOw	0.897*	0.948*	0.962**	0.964**	0.897*	0.917*			
RPw	0.874	0.957*	0.966**	0.943*	0.872	0.927*	0.996**		
TPCw	0.955*	0.832	0.930*	0.996**	0.972**	0.788	0.963**	0.945*	
TFCw	0.977**	0.794	0.931*	0.996**	0.989**	0.741	0.945*	0.923*	0.996**

*Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed)

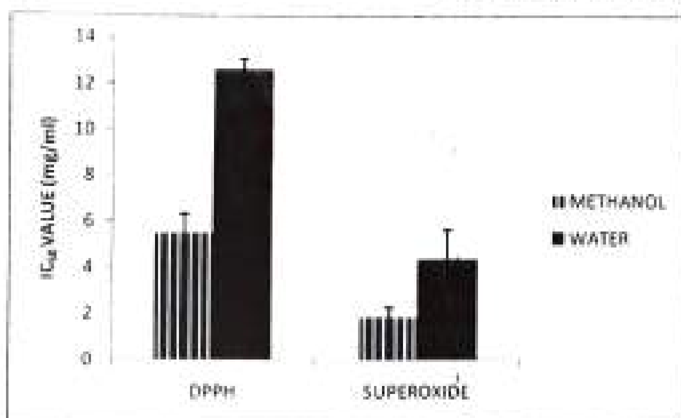

Figure 2: DPPH and Superoxide radical scavenging activity of MV leaves in aqueous decoction and methanol extract

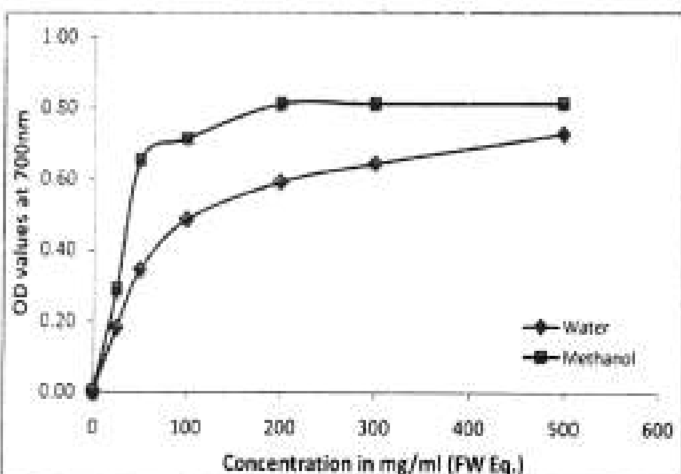

Figure 3: Reducing power activity of MV leaves in aqueous decoction and methanol extract

reddish violate colour appeared, the existence of triterpenoids was confirmed (Kumar *et al.*, 2009).

Test for cardiac glycoside

0.5 ml of leaves extracts were evaporated and dissolved in 1ml glacial acetic acid. One drop of 10% $FeCl_3$ was then added. 1ml of conc.H_2SO_4 was added by the side of the test tube. Appearance of brown colour ring at the interface indicated of presence of cardiac glycosides (Ngbede *et al.*, 2008).

Test for Flavonoids

To 1 ml of leaves extract, few drops of 10 % $FeCl_3$ solution were added. A green or blue colour indicated the presence of phenolic nucleus (Brain and Turner, 1975).

Test for tannin

0.5 ml leaves extract was added with 0.5 ml 1% lead acetate; a yellow colour precipitation indicated the presence of tannin (Kumar *et al.*, 2009).

Test for glycosides

0.5 ml leaves extract were added with 2ml of 50% HCl. The mixtures were hydrolyzed for 2 hrs on a water bath. After that 1ml pyridine, few drops of 1% sodium nitroprusside solution, and 5% NaOH solution were added. Pink to red colour designated the presence of

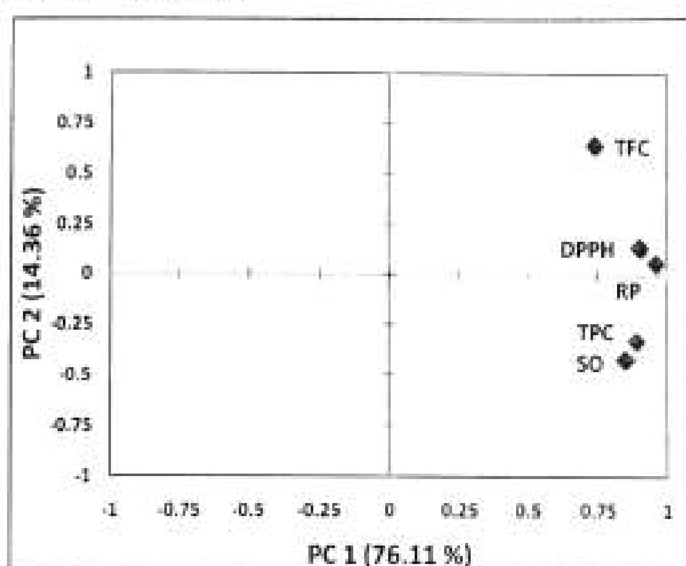

Figure 4: Principle component analysis with DPPH, SO, RP, TPC and TFC

glycosides (Kumar *et al.*, 2009).

Test for Saponins

2 ml of double distilled water was added with 1 ml of each leaves extract. Few drops of olive oil were added and agitated. Formation of soluble emulsion indicated the presence of saponin (Ngbede *et al.*, 2008).

Statistical analysis

The values are means of triplicate analysis of the samples (n=3) and were subjected to analysis of correlation co-efficient matrix using SPSS (Version 12.00) for drawing the relation between phytochemical properties and antioxidant attributes and MS Excel of Microsoft Office, 2007 was used for comparing the antioxidant attributes of methanolic and aqueous decoction extract of MV leaves. Smith's Statistical Package (Version 2.5) was used for determining the IC_{50} values of antioxidants and their standard error of estimates (SEE). In order to examine and visualize relationships between different phytochemicals and antioxidant traits, a principal component analysis (PCA) based on the correlation matrix was calculated using Multivariate Statistical Package (MVSP 3.1).

Results and Discussion

In this present study, MV leaves were evaluated for their antioxidant potential, considering the fact that antioxidant activity of the plant material is strongly dependent on the nature of extracting solvent due to the presence of different antioxidant compounds of varied chemical characteristics and polarities that may or may not be soluble in a particular solvent. The extractive values are useful to evaluate the chemical constituents present in the plant materials and also help in estimation of specific constituents soluble in a particular solvent (Ozarkar, 2005). According to Hsu *et al.*, 2006 the differences in the extractive values for the tested plant materials in different solvents might be due to the differences in the availability of extractable components, resulting from the varied chemical composition of plants. The extractive values obtained from aqueous

decoction of MV leaves and methanolic extract are 1.74% and 2.96% respectively. In our study, methanol solvent system was found to be more efficient in recovery of antioxidant compounds from MV leaves than aqueous decoction, thus offering higher extractive yield. Our findings suggest that higher extractive yield in methanol as compared to aqueous decoction may be due to more non-polar components in experimental plant sample. Similar result was found by Stanojevic *et al.* (2009) where the magnitude of extract yield of *Hieracium pilosella* leaf in methanol solvent system was higher than in water.

The methanolic and aqueous extracts were subjected to screening for their possible antioxidant activity. Four complementary test systems, namely DPPH free radical-scavenging, reducing power, and total flavonoid and phenol concentration, were used for the analysis.

DPPH has been extensively used as a free radical to evaluate reducing substances and is a useful reagent for investigating the free radical scavenging actions of compounds (Duan *et al.*, 2006). The scavenging capacity of aqueous decoction and methanol extracts of MV leaves were evaluated by measuring the decrease in DPPH radical absorption. The highest DPPH radical scavenging activity was detected in methanol extract (IC_{50} 5.4mg/ml) of MV leaves, when compared with aqueous decoction (IC_{50}12.6 mg/ml) (Figure 2). Methanol extract have greater antioxidant activity than the aqueous extracts may be due to the presence of the active less polar compounds in the leafy part of the vegetable that may dissolve only in the methanol, but not exactly in aqueous system. Similar result was found by Vadivelan *et al.*, (2009) where methanol extract of *Rubus ellipticus* root exhibited stronger radical scavenging activity than water, chloroform, ethyl acetate and petroleum ether.

In our study, the aqueous decoction and methanolic extracts of MV leaves were screened for their $O_2^{\cdot-}$ scavenging activity using PMS-NADH-NBT assay. In the PMS/NADH-NBT system, $O_2^{\cdot-}$ derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The effect of methanolic extracts of MV leaves was highest with IC_{50} value of 1.76 mg/ml than aqueous decoction with IC_{50} value of 4.33 mg/ml (Figure 2).

The reducing power was measured by the conversion of a Fe^{3+}/ferricyanide complex to the ferrous form. The reducing power was increased by increasing the amount of extract and the reducing capacity of compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al.*, 1995). The reducing power of the aqueous decoction and methanolic extracts of leaves was examined as a function of their concentration. The reducing capacity of aqueous decoction and methanolic extract is illustrated in Figure 3. The methanolic extract of MV leaves has more reducing power than aqueous decoction. The reducing ability of both extract was dose-dependent; the results indicated the presence of more hydrophobic phenolics in methanolic extracts, the values being directly correlated with reducing capacities.

The highest content of total phenol, total flavonoid and DPPH scavenging activities were found in methanol extracts of MV leaves. According to Ghasemzadeh *et al.*, (2010) it seems that the yield and efficiency of the phenolics extraction depends on the type and kind of the solvent as well as on the flavonoids, which is being isolated. For total phenolics and flavonoids extraction from ginger parts, methanol was found to be more efficient as compared to acetone and chloroform. Similar results were obtained from our study where methanol was found to be more effective for extracting phenolic compounds as these compounds are semipolar in nature and sometimes esterified with other conjugates. The higher total phenolic content of plants extracts resulted in higher antioxidant activity (Wong *et al.*, 2006). The total phenol contents of aqueous decoction and methanol extract of MV leaves were 0.53mg/g fresh weight tissue (FWT) and 1.73mg/g FWT respectively (Table 1).

Flavonoids are large family of polyphenolic components synthesized by plants. It was found that flavonoids functioned to reduce blood-lipid and glucose and to enhance human immunity (Atoui *et al.*, 2005). Flavonoids were also a kind of natural antioxidant capable of scavenging free superoxide radical, anti-aging and reducing the risk of cancer. The total flavonoid contents of aqueous decoction and methanol extract of MV leaves were 25.3μg/g FWT and 7.2μg/g FWT respectively. According to Winston (1999) , the leafy part of the vegetables contain the active component which consist of the flavonoid, terpenoid, lignan, sulphide, polyphenol, carotenoid, caumarin, saponin, curcumin and sterol. Aqueous decoction of MV leaves and methanolic extracts were also evaluated for semi-quantitative determination of major phyto-constituents i.e. steroid, alkaloid, cardiac glycoside, flavonoids, tannins, glycosides and saponin. The results of phytochemical analysis of MV leaves are presented in Table 2. Phytochemical screening of the MV leaves revealed some differences in the phytochemical constituents extracted in methanol and hot water solvent system. Phytochemical screening showed the presence of steroid, cardiac glycosides, flavonoid, tannin, glycosides and saponin in both aqueous decoction and methanolic extract of MV leaves whereas negative result was observed for alkaloid and triterpenoid test for both MV extracts.

Recently several authors have evaluated the relationship between antioxidant activity and defensive secondary metabolites like phenolics and flavonoids (Zhou *et al.*, 2006; Ninfali *et al.*, 2005; Aires *et al.*, 2011). In this study, Pearson's correlation coefficient was analyzed to determine the relationship between free-radical scavenging activity and metabolic components of MV. As shown in Table 3, overall strong correlation between the mean values of total phenol content and IC_{50} values of DPPH and superoxide free radical scavenging capacity was observed. Total flavonol content of both aqueous and methanolic extracts were also highly correlated with antioxidant activity. From Table 1,

reducing potential of both methanol and aqueous extracts were observed to be correlated with DPPH and superoxide radical scavengers. This could be explained from the basic concept that antioxidants are reducing agents and are capable of donating a single electron or hydrogen atom for reduction. However, not all reducing agents are antioxidants. Our investigation shows that reducing potential of MV were more associated with superoxide scavenging than DPPH'; indicating that compounds present in the polar extracts, capable of reducing DPPH radicals were also able to reduce ferric ions. A strong correlation between DPPH radical scavengers and reducing potential was determined with edible tropical plants (Wong *et al.*, 2006). Significant correlations were also found between total phenol content and ferric reducing antioxidant power in different genotypes of mulberry (Ozgen *et al.*, 2009). These results seem to confirm the idea that herbal polyphenols in fact are very important as free radical scavengers and extractable with polar solvents like methanol or water.

Principle component analysis (PCA) was performed in the classification of antioxidant activities of selected plant extracts (Wong *et al.*, 2006). In present investigation, PCA was performed to understand how the five parameters, namely, DPPH free radical scavenging ability (DPPH'), superoxide scavenging (SO), ferric ion reducing power (RP), total phenol (TPC) and flavonol content (TFC) contribute to antioxidant activity of plant extracts. The loadings plot (Figure 3) was used to gain an overview of the significance among antioxidant assays and the quantity of phytochemicals. The loadings of first and second principal components (PC1 and PC2) accounted for 76.11 and 14.36% of the variance, respectively. The most significant component, RP contributed the largest variation of approximately 24%, while the DPPH and TPC accounted for approximately 21% each of the total variation on PC1. Conversely, TFC was the most emphasized parameter (57% approximately) of PC2. But interestingly, superoxide did not contribute much on these two components, as it only indicated the ability of these extracts to control superoxide radicals under crisis condition, and is not directly responsible for antioxidant capacity in normalized system. Figure 3 shows that all five parameters were positively loaded on PC1 with significant quantity (squared cosine of variables are from 0.925 to 0.552), whereas in PC2 only TFC exhibited high quantum of positive loading. The results from PCA suggested that DPPH & RP may act with same components, as they are present in one cluster, while the other cluster having TPC & SO contributed in same fashion. From PCA score, it may also be proposed that TFC may be more useful marker of antioxidant activity in the leaves of MV, as the existence of one or more hydroxyl groups in A & B ring of flavonoids may directly involve in the transfer of hydrogen atom or an electron for detoxification of free radicals during oxidative stress.

Conclusion:

The results of this study revealed that the leaves of MV possess potential antioxidant activity. However, extracting solvent significantly affected antioxidant activity of MV leaves. Methanolic extracts of leaves exhibited stronger antioxidant activity in comparison to the aqueous decoction of MV leaves. It seems that yield and efficiency of the natural antioxidants depend on the type and kind of the solvent used. For total phenolics extraction from MV leaves, methanol was found to be more efficient compared to aqueous decoction but significantly for flavonoid extraction, aqueous decoction was more effective. The present data would certainly help to establish the effectiveness of the tested plant material as a potential source of natural antioxidants to be used for nutraceutical and functional food applications. Further research is required to identify individual components forming antioxidative system which could be exploited as cost effective food addictives for human health.

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