In Vitro Antioxidant Activity of *Cucurbita Maxima* Aerial Parts

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ABSTRACT

Introduction: Free radical induced oxidative stress is involved in the pathogenesis of various diseases and disorders. Antioxidants play an important role against this oxidative stress to protect our body. The present study was carried out to evaluate the *in vitro* antioxidant properties of methanol extract of *C.maxima* aerial parts (MECM). **Methods:** MECM was assayed on different *in vitro* free radical models like, DPPH, nitric oxide, superoxide, hydrogen peroxide and lipid peroxide radical models. Reductive ability of the extract was also tested by the complex formation with potassium ferricyanide. Further total phenolic and flavonoid contents of the crude extract were also measured. Butylated hydroxy toluene was taken as standard. **Result:** The extract showed good dose dependent free radical scavenging activity in all the models. Reductive ability was also found to increase with increase in extract concentration. Determination of total phenolic and total flavonoids content showed that 1 gm of dry extract contains 66.70±3.60 mg equivalent of pyrocatechol and 26.50±1.40 mg equivalent of quercetin. **Conclusion:** All the results of the *in vitro* antioxidant assays revealed potent antioxidant and free radical scavenging activity of the aerial part of *C.maxima*, equivalent to that of standard BHT and this antioxidant property may be attributed to its high phenolic and flavonoid contents.

Keywords: *Cucurbita maxima*, Cucurbitaceae, DPPH, free radicals, Lipid peroxidation, oxidative stress.

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INTRODUCTION

Oxygen derived free radicals such as superoxide, hydrogen peroxide, hydroxyl radicals are collectively known as reactive oxygen species (ROS).During normal physiologic condition, ROS are continuously produced in the aerobic cells and removed by endogenous antioxidant defense mechanism of the cell. But, under pathologic condition the balance between ROS and antioxidant defense mechanism is lost.[1] Overproduction of ROS and other free radicals then can damage cellular proteins, carbohydrates, lipids and DNA and may thus lead to oxidative stress which in turn results in a variety of diseases, such as liver cirrhosis, inflammation, atherosclerosis, diabetes, cancer, neurodegenerative disease, nephrotoxicity and also the aging process. Antioxidants have the potential to prevent these oxidative damages and thereby minimize the homeostatic disturbances.[2-6]

Many plants contain substantial amounts of antioxidants and 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.[7,8]

The plant *Cucurbita maxima* Duchesne (commonly known as pumpkin) belongs to the family Cucurbitaceae and is widely cultivated throughout the world for use as vegetable as well as medicine. Both of its fruits and the aerial parts are commonly consumed as vegetable. It is a large climbing herb, annual or perennial. Its aerial part consists of flexible succulent stem with trifoliate leaves. [9] The plant has been used traditionally as medicine in many countries such as China, India, Yugoslavia, Brazil and America.[10-12] Traditionally it is used in most countries as antidiabetic, antitumor, antihypertensive, anti-inflammatory, immunomodulatory and antibacterial agents.[13] Several *in vitro* and *in vivo* studies with crude pumpkin fruit extract as well as various purified fractions, including proteins and polysachharides, have explored its antitumor, antidiabetic and other medicinal values.[10,14] Popularity of pumpkin in various traditional system of medicine for several ailments focused the investigators'

attention on this plant. The present study was therefore carried out to investigate the antioxidant potential of methanol extract of *C. maxima* aerial parts (MECM) on different *in vitro* models.

MATERIALS AND METHODS

Chemicals

1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) was obtained from Sigma Chemicals, USA. Nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), reduced nicotinamide adenine dinucleotide (NADH), sodium nitroprusside, napthyl ethylene diamine dihydrochloride, ascorbic acid, trichloroacetic acid (TCA), thiobarbituric acid (TBA), potassium ferricyanide [$K_3Fe(CN)_6$] , Folin-Ciocalteu's phenol reagent (FCR) were purchased from Sisco Research Laboratories Pvt. Ltd., Mumbai, India. All other chemicals and solvents used were of analytical grade.

Plant Material

The aerial parts of *C.maxima* were collected in June 2009, from Khardah, West Bengal, India and identified by the Botanical Survey of India, Howrah, India. A voucher specimen (P/CM/2/09) was retained in our laboratory for further reference.

Preparation of Plant Extract

The aerial parts were dried and powdered in a mechanical grinder. The powdered material was extracted with methanol using soxhlet apparatus. This extract was filtered and concentrated in *vacuo* in a Buchi evaporator, R-114 and kept in a vacuum dessicator until use. The yield was 11.49% w/w with respect to dried powder. This methanol extract was used for the present study.

Preliminary Phytochemical Screening

Preliminary phytochemical screening was carried out by following the standard procedures.[15]

In vitro Antioxidant Studies

Various concentrations of MECM (10-320 μg/ml in methanol) were used for the antioxidant studies on different *in vitro* models. For reductive ability study, 100-800 μg/ml concentration of the extract was used. Butylated hydroxy toluene (BHT) was used as standard.

Determination of DPPH Radical Scavenging Activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was measured using the method of Cotelle *et al*[16] with some modifications. 3 ml of reaction mixture containing 0.2 ml of DPPH (100 μM in methanol) and 2.8 ml of test or standard solution of various concentrations was incubated at 37°C for 30 min and absorbance of the resulting solution was measured at 517 nm using Beckman model DU-40 spectrophotometer. The percentage inhibition of DPPH radical was calculated by comparing the results of the test with those of the control (not treated with extract) using the formula:

$$\text{Percentage inhibition} = \frac{(\text{Absorbance of control} - \text{Absorbance of test})}{\text{Absorbance of control}} \times 100$$

Determination of Nitric Oxide (NO) Scavenging Activity

At physiological pH, sodium nitroprusside generates nitric oxide, which interacts with oxygen to produce nitrite ions, which can be measured by the Griess reaction[17]. 1ml of 10 mM sodium nitroprusside was mixed with 1 ml of test or standard solution of different concentrations in phosphate buffer (pH 7.4) and the mixture was incubated at 25°C for 150 min. From the incubated mixture, 1 ml was taken out and 1 ml of Griess' reagent (1% sulphanilamide, 2% o-phosphoric acid and 0.1% naphthyl ethylene diamine dihydrochloride) was added to it. Absorbance of the chromophore formed by the diazotization of nitrite with sulfanilamide and subsequent coupling with naphthyl ethylene diamine dihydrochloride was read at 546 nm and percentage inhibition was calculated by comparing the results of the test with those of the control using the above formula.

Determination of Superoxide (SO) Radical Scavenging Activity

Superoxide anion scavenging activity was measured according to the method of Robak and Gryglewski[18] with some modifications. All the solutions were prepared in 100 mM phosphate buffer (pH 7.4). 1ml of nitroblue tetrazolium (NBT, 156 μM), 1 ml of reduced nicotinamide adenine dinucleotide (NADH, 468 μM) and 3 ml of test/ standard solution were mixed. The reaction was initiated by adding 100 μl of phenazine methosulphate (PMS, 60 μM). The reaction mixture was incubated at 25°C for 5 min,

followed by measurement of absorbance at 560 nm. The percentage inhibition was calculated from the above formula.

Determination of Hydrogen Peroxide (H_2O_2) Scavenging Activity

The hydrogen peroxide scavenging ability of the extract was determined according to the method of Ruch et al[19]. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Extract or standards (of different concentrations) in phosphate buffer (3.4 ml) was added to the H_2O_2 solution (0.6 ml, 40 mM). The absorbance of the reaction mixture was recorded at 230 nm after 10 min against a blank solution of phosphate buffer. Percentage of H_2O_2 scavenging was calculated using the above formula.

Determination of Lipid Peroxide (LPO) Radical Scavenging Activity

Rat liver homogenate was used as the source of polyunsaturated fatty acids for determining the extent of lipid peroxidation. Reaction mixture (0.5 ml) containing rat liver homogenate (0.1 ml, 25% w/v) in Tris–HCl buffer (40 mM, pH 7.0), KCl (30 mM), ferrous ion (0.16 mM) and ascorbic acid (0.06 mM) were incubated at 37°C for 1 h in the presence or absence of the extracts or standards. The lipid peroxide formed was measured according to the method of Ohkawa *et al*[20]. Incubation mixtures were treated with sodium dodecyl sulphate (SDS; 8.1%, 0.2ml), thiobarbituric acid (TBA; 0.8%, 1.5 ml) and acetic acid (20%, 1.5ml). The total volume was then made up to 4 ml with distilled water and kept on water bath for 30 min. After cooling, 1 ml of distilled water and 5 ml of a mixture of n-butanol and pyridine (15:1 v/v) were added and centrifuged at 4000 rpm for 10 min. The absorbance of the organic layer, containing the colored TBA-MDA complex, was measured at 532 nm. The percentage inhibition of lipid peroxidation was determined by comparing the results of the test compound with those of the control, using the formula mentioned above.

Determination of Reductive Ability

Reducing power of the extract and the standard was determined on the basis of the ability of their antioxidant principles to form colored complex with potassium ferricyanide, trichloro acetic acid (TCA) and $FeCl_3$, which is measured at 700 nm[21]. 1 ml of different concentrations of the extract or standard solution was mixed with potassium ferricyanide (2.5 ml, 1%) and 2.5 ml of phosphate buffer (pH 6.6). The mixture was incubated at 50°C for 20 min. 2.5 ml TCA (10%) was added to it and centrifuged at 3000 rpm for 10 min. 2.5 ml of supernatant was taken out and to this 2.5 ml water and 0.5 ml $FeCl_3$ (0.1%) were added and absorbance was measured at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power.

Determination of Total Phenolic Content

The amount of total phenolic compounds in MECM was determined using Folin-Ciocalteu's reagent and sodium carbonate solution and the absorbence was measured at 760 nm[22]. A calibration curve of standard pyrocatechol was prepared and the results were expressed as mg of pyrocatechol equivalents /g of dry extract.

Determination of Total Flavonoid Content

The total flavonoid content of MECM was determined spectrophotometrically[23]. Briefly 0.5 ml of 2% aluminium chloride in ethanol was mixed with same volume of extract (1.0 mg/ml). Absorption readings at 415 nm were taken after 1 h against a blank (ethanol). The total flavonoid content was determined using a standard curve with quercetin (0-50 mg/L). The mean of three readings was used and expressed as mg of quercetin equivalents/ g of dry extract.

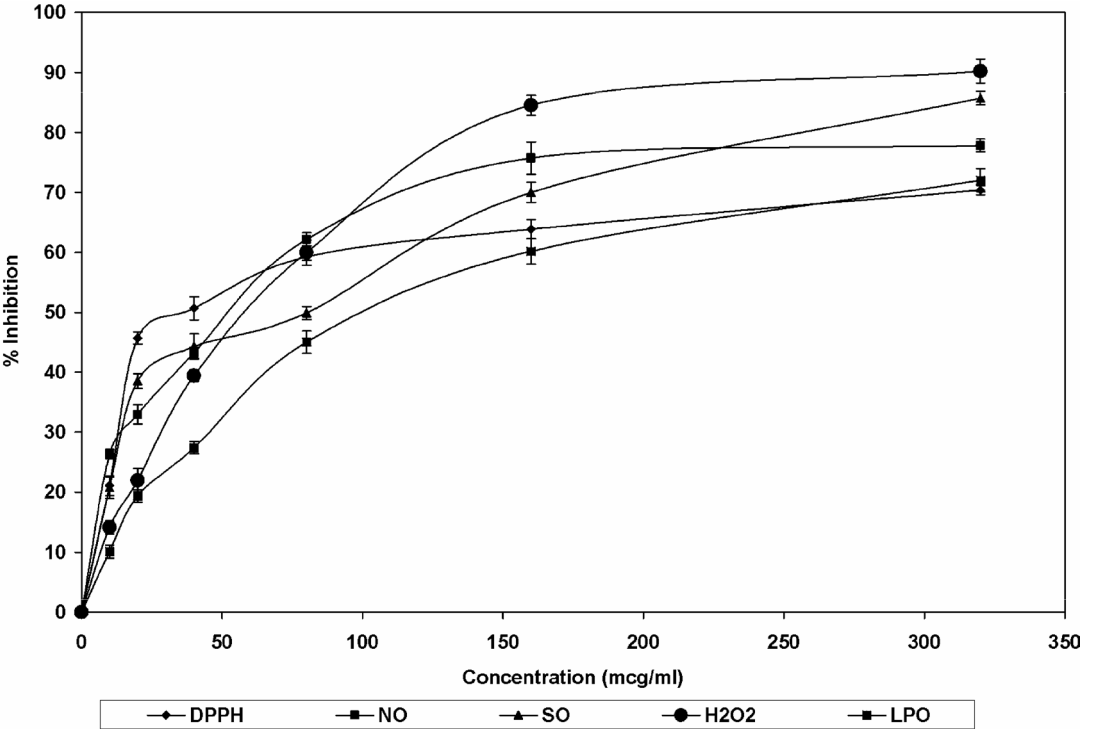
Statistical Analysis

Data were expressed as mean ± SEM of triplicate determinations. Linear regression analysis was used to calculate IC_{50} values wherever needed.

RESULTS

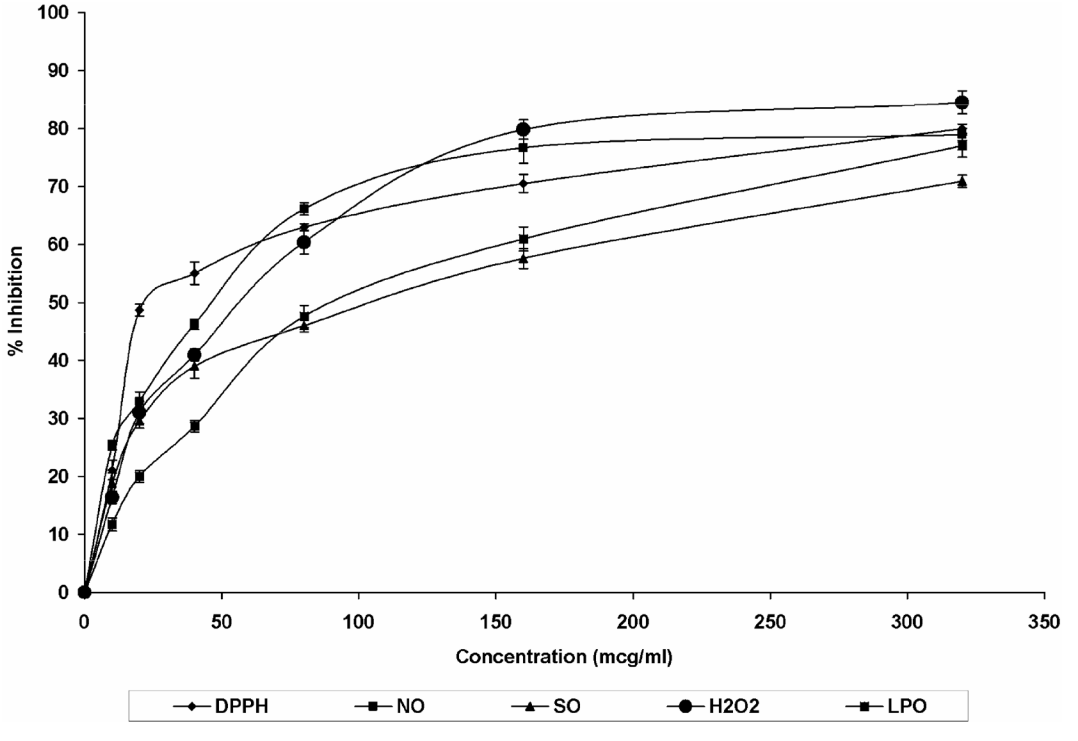
Preliminary phytochemical screening of MECM revealed the presence of polyphenolics, flavonoids, glycosides, triterpinoids and carbohydrates. The extract showed good antioxidant activity in all *in vitro* free radical scavenging assays. Percentage inhibition in various models, viz. DPPH, NO, SO, H_2O_2 and LPO is shown in Fig.1 and was found to be quite equivalent to that of the standard, BHT (Fig.2). IC_{50} (Inhibitory concentration at which there is 50% reduction in free radical reaction) values have been presented in Table.1. Reducing power of the extract was

dose dependent which was indicated from the increase in absorbance with the increase in concentration of the extract (Fig.3). Determination of total phenolic content and total flavonoids showed that 1 gm of dry extract contained 66.70±3.60 mg equivalent of pyrocatechol and 26.50±1.40 mg equivalent of quercetin.



Values are mean ± SEM of triplicate determinations

Figure 1. Effect of methanol extract of *C.maxima* aerial parts (MECM) on different *in vitro* free radical models.



Values are mean ± SEM of triplicate determinations

Figure 2. Effect of Butylated hydroxy toluene (BHT) on different *in vitro* free radical models.

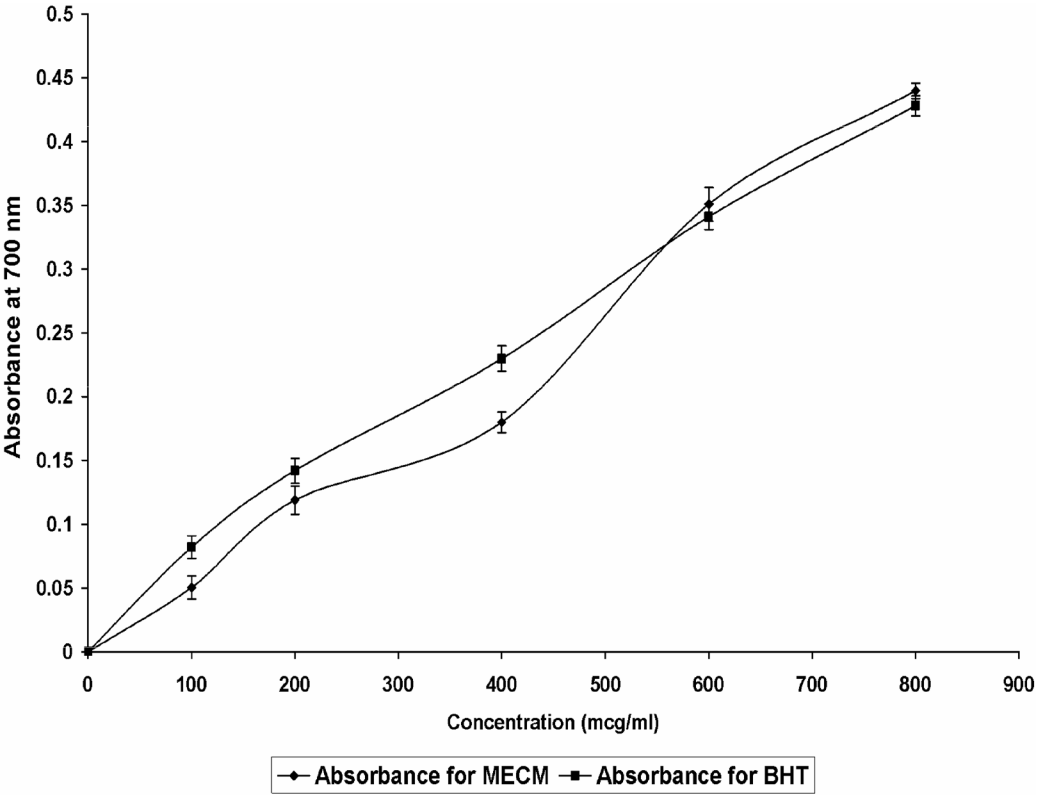


Figure 3. Reductive ability of methanol extract of *C.maxima* aerial parts (MECM) and Butylated hyrdoxy toluene (BHT).

Table 1. Free radical scavenging activity of methanol extract of *C.maxima* aerial parts (MECM) and Butylated hydroxy toluene (BHT) on different in vitro models

In vitro models	IC_{50} Values (µg/ml)	
	MECM	BHT
DPPH	38.05±1.12	22.05±4.10
NO	51.00±2.55	52.40±3.33
SO	80.35±3.50	120.50±5.75
H_2O_2	54.06±2.89	23.85±2.95
LPO	96.50±4.06	98.86±3.33

DPPH: Diphenyl-2-picryl hydrazyl; NO: Nitric oxide; SO: Super oxide; H_2O_2: Hydrogen peroxide; LPO: Lipid peroxide.
% Inhibition = (Absorbance of control – Absorbance of test) × 100/ Absorbance of control
IC_{50} Value: Inhibitory concentration at which there is 50% reduction in free radical reaction
Values are mean ± SEM of triplicate determinations

DISCUSSION

Oxidative stress as a result of excessive free radical generation is the main underlying feature of various diseases and disorders. Antioxidants can protect the organism against this oxidative stress by terminating the chain reactions of free radicals in the system. However there are certain restrictions on use of synthetic antioxidants, such as BHT, BHA, as they are suspected to be hepatotoxic and carcinogenic.[24,25] Natural antioxidants have therefore gained the momentum in the recent research studies.

DPPH radical scavenging assay is one of the most widely used methods for screening of antioxidant property of plant products[16]. DPPH is a stable nitrogen centered free radical and can easily abstract an electron or hydrogen radical from the suitable reducing agents to become a stable diamagnetic molecule. The unpaired electron of DPPH thus gets paired off forming the corresponding non-radical hydrazine[26]. The radical scavenging property of the sample was determined by measuring the decrease in absorbance of DPPH. The dose dependent inhibition of DPPH radical indicates that MECM can reduce DPPH radical in a stoichiometric manner and this radical scavenging effect may be due to its hydrogen donating property.

Nitric oxide is an important chemical mediator, involved in the number of physiological processes and regulation of cell mediated toxicity. However, excess nitric

oxide can react with oxygen to generate nitrite and peroxy nitrite anions, which act as free radicals and may result in various deleterious effects in the cells. Therefore the production of nitric oxide should be regulated as much as possible.[27] In the present study, the antioxidant principles in the MECM was found to compete with oxygen to react with nitric oxide and thus inhibit the generation of the anions in dose dependent manner.

Superoxide anion plays an important role in the formation of various ROS which induce oxidative damage in lipids, proteins and DNA and thereby precipitates various pathological conditions.[28] In the present study, superoxide, derived from dissolved oxygen by PMS-NADH coupling reaction, reduces the yellow dye (NBT^{2+}) to produce the blue formazan which is measured spectrophotometrically. Antioxidants are able to inhibit the process by inhibiting the generation of superoxide in the *in vitro* reaction mixture[29]. Dose dependent scavenging effect of MECM was found in the present study, indicating the protective effect of the extract in oxidative stress.

Hydrogen peroxide is a weak oxidizing agent and itself is not much reactive. However it can cross cell membrane easily and inside cell it may give rise to hydroxyl radical formation, which is deleterious for the cell.[3] Thus excess hydrogen peroxide should not be accumulated in the cell. Dose dependent H_2O_2 scavenging activity of MECM, found in the present study, is therefore quite beneficial to reduce oxidative damage.

Lipid peroxidation is one of the oxidative conversion processes of polyunsaturated fatty acids to lipid peroxides and various carbonyl products such as malondialdehyde (MDA), which are highly cytotoxic[30]. Increased lipid peroxidation can also impair membrane functions by decreasing membrane fluidity and changing the activity of membrane bound enzymes and receptors. Initiation of the lipid peroxidation by ferrous sulphate takes place either through ferryl-perferryl complex [Fe^{2+}- $O_2 \leftrightarrow Fe^{3+}$-$O_2^-$] or through $OH^\bullet$ radical by Fenton's reaction [Fe^{2+}+ $H_2O_2 \rightarrow Fe^{3+}$+ $OH^\bullet$+ OH^-][1]. In the present study, MECM was found to inhibit the process of lipid peroxidation and thereby reduced the free radical toxicity that strongly supports its antioxidant activity.

The reductive ability of a compound may serve as a significant indicator of its potential antioxidant activity. For the measurement of the reductive ability, in the present study, the Fe^{3+}-Fe^{2+} transformation was investigated in the presence of the MECM. Presence of reductants causes the reduction of the Fe^{3+}/ferricyanide complex to the Fe^{2+} form[31]. Results depict that the reducing power of *C. maxima* increases with increasing concentration which implies that the extract is capable of donating H atoms in a dose dependent manner.

CONCLUSION

Thus the present study shows the significant antioxidant potential of MECM in all *in vitro* assays. Further, MECM was found to possess significant amount of total phenolic and flavonoid content. Antioxidant property of many polyphenolic compounds, particularly, flavonoids have been reported in other investigations.[7,32] Potent antioxidant activity of MECM in the present study may therefore be attributed to its high phenolic and flavonoid content. Antioxidant property is widely used as a parameter for medicinal bioactive components. Many studies have shown that the antioxidant activities of the natural products are closely related to their biofunctionalities.[33,34] Potent antioxidant property of MECM, revealed in the present investigation may therefore be important in the future research works to study the underlying mechanism of its various medicinal properties. Further research can also explore the particular antioxidant principle(s) from the *C. maxima* aerial parts extract which can be one of the potent lead compound(s) from the arsenal of natural products.

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