

# Free radical scavenging activity and antioxidative potential of various solvent extracts of *Mussaenda macrophylla* Wall: An *in vitro* and *ex vivo* study

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## ABSTRACT

This study investigates the phytochemical composition, free radical scavenging activities, and antioxidative potential of various extracts of *Mussaenda macrophylla*. Phytochemicals such as alkaloids, cardiac glycosides, saponins, steroids, tannins, and terpenoids were found to be present in various extracts of *M. macrophylla*. Aqueous extract of *M. macrophylla* has the highest total phenolic ( $387.61 \pm 14.10$  mg gallic acid equivalent/g) and flavonoid ( $5,761.65 \pm 38.5$  mg quercetin equivalent/g) contents. The antioxidative potential of *M. macrophylla* extracts was measured by their ability to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH), superoxide anions ( $O_2^{\bullet-}$ ), and 2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) in a cell free system. The aqueous extract of *M. macrophylla* possessed the highest scavenging activities for DPPH,  $O_2^{\bullet-}$ , and ABTS with  $IC_{50}$  of  $25.92 \pm 0.33$   $\mu$ g/ml,  $4.12 \pm 0.94$   $\mu$ g/ml, and  $17.20 \pm 0.50$   $\mu$ g/ml, respectively. Furthermore, the scavenging activities of the aqueous extract of *M. macrophylla* against ABTS and  $O_2^{\bullet-}$  were found to be more effective than ascorbic acid which was used as standard. The total reducing power of *M. macrophylla* extracts was also determined by measuring the transformation of  $Fe^{3+}$  into  $Fe^{2+}$  and the methanolic extract was found to exhibit the highest reducing power. The aqueous extract of *M. macrophylla* showed the highest inhibitory activities against mice erythrocyte hemolysis and lipid peroxidation in the liver homogenate with an inhibition rate of 80.53% and 65.33%, respectively.

## INTRODUCTION

Free radicals are essential bio-regulatory molecule required for several physiological functioning of the body such as regulation of gene expression, immune response, and cellular growth (Droge, 2002; Tothhawng *et al.*, 2013), representing an important part of aerobic life (Tiwari, 2001). However, due to their highly reactive property, over-production of the free radicals including superoxide anion radicals ( $O_2^{\bullet-}$ ), singlet oxygen ( $^1O_2$ ), hydrogen peroxide ( $H_2O_2$ ), and hydroxyl radicals ( $\bullet OH$ ) can hamper the body antioxidant defense systems leading to a condition called "oxidative stress". Oxidative stress is found to be closely associated with various lifestyle-related disorders

including neurodegenerative disorders, arthritis, coronary heart disease, inflammation, diabetes, and cancer (Maxwell, 1995; Stadtman, 1992; Valko *et al.*, 2006). Despite the presence of small antioxidant molecules (e.g., glutathione) and several antioxidant enzymes (e.g., glutathione peroxidase, glutathione reductase, catalase, superoxide dismutase) in our body, these agents are not sufficient to maintain the normal redox status during increased oxidative stress (Seifried *et al.*, 2007). Therefore, maintenance of optimal body function and redox homeostasis in cells may require exogenous antioxidants supplementation. Medicinal plants have been used for human healthcare and represent the mainstay of traditional systems of medicine. The importance of plants as natural sources of antioxidants and free radical scavengers has been reported earlier by various researchers (Auddy *et al.*, 2003; Poullain *et al.*, 2004; Siama *et al.*, 2018; Shantabi *et al.*, 2014). Many modern drugs used for the treatment of several diseases have been isolated from different medicinal plants (Cragg and Newman, 2013). Consumption of natural antioxidants, such as

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those derived from plants, has been reported to lower the risk of cancer and many lifestyle-related disorders (Gerber *et al.*, 2002).

Mizoram, a state in North-East India, with its rich biodiversity offers a number of medicinal plants. One such plant is *Mussaenda macrophylla*, a member of the Rubiaceae family, locally known as Vakep. *M. macrophylla* is a flowering shrub, endemic to south-east Asia, reported to occur in India, China, and Myanmar (Manandhar, 2002). *Mussaenda macrophylla* has been traditionally used for the treatment of various health problems such as coughs, chronic ulcer, diarrhea, dysentery, indigestion, cancers, and even for the treatment of snake bites (Rosangkima and Jagetia, 2015; Sharma *et al.*, 2001). Among the genus *Mussaenda*, *M. frondosa* (Siju *et al.*, 2010), *M. glabrata* (Menon and Sasikumar, 2011), and *M. roxburghii* (Aktar *et al.*, 2014; Islam *et al.*, 2015) have been reported to have multi-pharmaceuticals importance such as antioxidants, anti-inflammatory, and thrombolytic activity. Despite the extensive use of *M. macrophylla* as traditional medicine, there has been no scientific validation of their pharmaceutical property. Therefore, we aimed to investigate the free radical scavenging and antioxidative potentials of various solvent extracts of *M. macrophylla* both *in vitro* and *ex vivo*.

## MATERIALS AND METHODS

### Chemicals

Quercetin dihydrate, gallic acid, 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), nicotinamide adenine dinucleotide, 2-deoxyribose, phenazine methosulfate, nitro blue tetrazolium (NBT), disodium hydrogen phosphate, ferric chloride, sodium nitrite, potassium persulfate, and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) were obtained from HiMedia Laboratories Pvt., Ltd. (Mumbai, India). Thiobarbituric acid and 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals were obtained from Sigma Aldrich Inc (Louis, Germany). Folin-Ciocalteu's reagent, trichloroacetic acid (TCA), sodium hydroxide, sodium carbonate, ascorbic acid (ASA), and ferrous sulfate were purchased from SD fine-Chem Ltd. (Mumbai, India). Aluminum chloride and sodium dihydrogen phosphate were obtained from Merck Specialities Pvt., Ltd. (Mumbai, India). Ethylenediamine tetraacetic acid was obtained from Qualigens Fine Chemicals (Mumbai, India). Potassium ferricyanide was purchased from Loba Chemie Pvt., Ltd. (Mumbai, India).

### Collection of plant and preparation of extracts

*Mussaenda macrophylla* was collected from Kolasib District, Mizoram. Identification and authentication of the plant were done by the Department of Horticulture, Aromatic and Medicinal Plants, Mizoram University, Aizawl. The leaves were dried naturally in a well-ventilated and dark place at room temperature for up to 30 days and pulverized by using a mixer grinder. The dried powdered leaves were then sequentially extracted with petroleum ether, chloroform, methanol, and distilled water using the Soxhlet apparatus for a minimum of 40 cycles each. The liquid extracts were filtered and dried in a rotary evaporator (Buchi, Germany) under reduced pressure at 40°C for 5 hours. The extracts obtained were then collected and stored at 4°C until use. Henceforth, chloroform extract, methanol extract, and aqueous extract of *M. macrophylla* will be called as MMCE, MMME, and MMAE, respectively.

### Phytochemical analysis

Preliminary phytochemical screening was performed using standard methods (Bargah, 2015; Gangwar *et al.*, 2014; Muthukrishnan and Manogaran, 2018). Samples of different fractions of *M. macrophylla* were tested for the presence of cardiac glycosides, alkaloids, saponins, steroids, tannins, terpenoids, and phlobatannins. Results are expressed as (+) for the presence and (–) for the absence of phytochemical.

### Estimation of total phenolic content

The total phenolic content of *M. macrophylla* was estimated using standard method (Khan *et al.*, 2012). Briefly, 5 ml of Folin-Ciocalteu's reagent (diluted ten-fold) was mixed with 1 ml of *M. macrophylla* extracts (0.25–8.0 mg/ml). After 5 minutes of incubation, 4 ml of sodium carbonate (0.115 mg/ml) was added to the mixture. The mixture was then incubated in the dark at room temperature for 2 hours and the absorbance was recorded at 765 nm. Calibration curve was also prepared by mixing methanol solution of gallic acid (1 ml, 0.25–4.0 mg/ml) with the reagents above and absorbance was recorded at 765 nm using an ultraviolet-Visible spectrophotometer. The experiment was repeated three times and the total phenolic content was expressed as gallic acid equivalents (GAEs) mg/g of the dry extract.

### Estimation of total flavonoid content

Total flavonoid content of various extracts of *M. macrophylla* was estimated according to the method previously described (Khan *et al.*, 2012) with minor modifications. Briefly, 0.25 ml of *M. macrophylla* extracts (0.25–8.0 mg/ml) and standard quercetin solution was mixed with 75 µl of 5% (w/v) sodium nitrite solution and 1.25 ml of distilled water. After the addition of 150 µl of 10% (w/v) aluminum chloride, the solution was allowed to stand for 5 minutes followed by the addition of 0.5 ml of 1 M NaOH. The volume of the solution was made up to 2.5 ml using distilled water and mixed well. The absorbance was recorded immediately at 510 nm. The total flavonoid content of *M. macrophylla* was expressed in terms of quercetin equivalent (mg/g extract).

### Determination of free radical scavenging activity *in vitro*

The ability of different extracts of *M. macrophylla* to inhibit the generation of various free radicals was carried out as described below.

### DPPH radical scavenging activity

The scavenging activity of *M. macrophylla* extracts for DPPH radical was determined according to Lahlminghlu and Jagetia (2018) with minor modifications. Briefly, 0.5 ml of various extracts of *M. macrophylla* (1–400 µg/ml) was mixed with 1 ml of methanol solution of 0.1 M DPPH followed by 30 minutes incubation in the dark. The absorbance of the solution at 523 nm was compared with the control. The scavenging activity of the plant extract against DPPH was expressed as IC<sub>50</sub> which is the concentration (µg/ml) of extract at which 50% of the DPPH radicals were inhibited. ASA was used as the standard. The test was repeated at all concentrations of each sample in triplicate. The scavenging activity was then calculated based on the percentage of DPPH radicals scavenged using the formula:

$$\text{Scavenging (\%)} = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100$$

Where  $A_{\text{blank}}$  is the absorbance of the control (solution containing all the reagents except the plant extracts) and  $A_{\text{sample}}$  is the absorbance of the solution containing the plant extract.

#### Superoxide radical scavenging activity

Superoxide scavenging activity was determined by the NBT reduction method (Lalhmingshlu and Jagetia, 2018) with minor modifications. In brief, the reaction mixture was prepared using 0.2 ml of NBT [1 mg/ml in dimethyl sulfoxide (DMSO)] and 0.6 ml of plant extract (1–800  $\mu\text{g/ml}$ ). Then, the volume of the mixture was made up to 2.8 ml using 2 ml of alkaline DMSO (1 ml DMSO in 5 mM NaOH). The absorbance of the mixture was recorded at 560 nm and pure DMSO was used as blank. ASA served as the standard and the ability of *M. macrophylla* extracts to scavenge the superoxide radical was calculated.

$$\% \text{ scavenging} = (A_c - A_o/A_o) \times 100$$

Where  $A_o$  is the absorbance without plant extract and  $A_c$  is the absorbance with the plant extract.

#### ABTS radical scavenging activity

The scavenging activity of *M. macrophylla* against ABTS was determined using the method of Khan *et al.* (2012). Briefly, 5 ml each of 7 mM ABTS and 2.45 mM potassium persulfate were mixed for a stock solution. A stock solution was then incubated at room temperature in the dark for 12 hours so as to yield a dark-colored solution that contains  $\text{ABTS}^{+\cdot}$  radicals. A freshly prepared working solution consists of a stock solution diluted with 50% methanol having an initial absorbance of 0.70 ( $\pm 0.02$ ) at 745 nm.  $\text{ABTS}^{+\cdot}$  radicals scavenging activity was then assessed by mixing 150  $\mu\text{l}$  of different fractions of various extracts of *M. macrophylla* (1–200  $\mu\text{g/ml}$ ) with 1.5 ml of ABTS working solution. The decrease in absorbance was measured immediately at 745 nm. The test was repeated at all concentrations of each sample in triplicate. ASA served as the standard. The scavenging activity of the plant extract was then calculated using the formula:

$$\text{Scavenging (\%)} = [(A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}] \times 100$$

Where  $A_{\text{blank}}$  is the absorbance of the control (solution containing all the reagents except the plant extracts) and  $A_{\text{sample}}$  is the absorbance of the solution containing the plant extract.

#### Reducing power

The reducing power of *M. macrophylla* extracts was estimated using the method given by Adjimani and Asare (2015) with minor modifications. Briefly, 2.5 ml each of 0.2 M phosphate buffer (pH—6.6) and 1% potassium ferricyanide solution were mixed with *M. macrophylla* extracts (1–400  $\mu\text{g/ml}$ ; dissolved in their respective solvent). After incubation of the mixture at 50°C for 20 minutes, 2.5 ml of 10% TCA was added. The mixture was then centrifuged at 3,000 rpm for 10 minutes. Equal volume of distilled water was added to the supernatant followed by 0.5 ml of 1% ferric chloride solution. Absorbance of the mixture was measured at 700 nm. The increase in absorbance indicated increasing reducing power of the extract.

## Ex vivo antioxidant assay

### Anti-hemolytic activity

The inhibition of mice erythrocyte hemolysis by various extracts of *M. macrophylla* was measured to determine their antioxidative potential (Zhou *et al.*, 2014). Blood was collected from Swiss albino mice of the same age group (10–12 w) and body weights (25–27 g) by heart puncture in a heparinized tube. The mice erythrocyte hemolysis was induced with  $\text{H}_2\text{O}_2$  that serve as a free radical initiator. A mixture was prepared by adding 0.5 ml of 5% (v/v) suspension of red blood cells (RBC) in phosphate buffered saline (PBS), 0.4 ml (0.5 mg/ml) of different extracts of *M. macrophylla*, and 100  $\mu\text{l}$  of 1 mol/L  $\text{H}_2\text{O}_2$ . The reaction mixture was gently mixed while being incubated at 37°C for 3 hours. It was then diluted with 4 ml of PBS and centrifuged at 2,000 rpm for 10 minutes. The supernatant was collected and absorbance was recorded at 540 nm. The rate of inhibition of erythrocyte hemolysis was then calculated.

$$\text{Inhibition rate (\%)} = [1 - (A1 - A2)/A_o] \times 100$$

Where  $A_o$  is the absorbance of the control, A1 is the absorbance of the solution containing the plant extract, and A2 is the absorbance without RBC.

### Inhibition of lipid peroxidation

Lipid peroxidation inhibitory potential of *M. macrophylla* extracts was measured according to the method given by Rajinder *et al.* (2010) using mice liver. The liver was excised from Swiss albino mice and 1% liver homogenate was prepared and centrifuged at 3,000 rpm at 4°C for 10 minutes. 0.5 ml of supernatant was mixed with 0.5 ml (0.5 mg/ml) of *M. macrophylla* extracts, 0.25 ml each of 0.5 mol/L  $\text{FeCl}_2$  and  $\text{H}_2\text{O}_2$ , and incubated at 37°C for 1 hour. Absorbance was measured at 535 nm and the rate of inhibition of lipid peroxidation was calculated using the formula:

$$\text{Inhibition rate (\%)} = [1 - (A1 - A2)/A_o] \times 100$$

Where  $A_o$  is the absorbance of the control, A1 is the absorbance of the solution containing the plant extract, and A2 is the absorbance without liver homogenate.

## Animals

Colony of inbred Swiss albino mice is being maintained under standard environmental conditions of temperature (22°C  $\pm$  5°C) and light (12 hours of light and dark, respectively) (Frontier Euro Digital Timer, Taiwan) at the Animal House, Department of Zoology, Mizoram University, India. The animals were having free access to food and water. Caring and handling of animals were carried out based on the guidelines given by WHO, Geneva, Switzerland. The study was approved by the Institutional Animal Ethical Committee, Mizoram University, India (No. MZU-IAEC/2018/09) and CPCSEA, New Delhi, India (Registration No. 1999/GO/ReBi/S/18/CPCSEA).

## Statistical analysis

Results are expressed as mean  $\pm$  standard error of mean. One-way analysis of variance was performed to test the significant variations on phytochemical contents and the antioxidative potential of various extracts followed by Tukey



multiple comparisons of means. The  $IC_{50}$  values were calculated by plotting the % inhibition of free radicals against the log doses using GraphPad Prism software version 6.0. A  $p$ -value of less than 0.05 was considered statistically significant.

## RESULTS AND DISCUSSION

### Phytochemical analysis

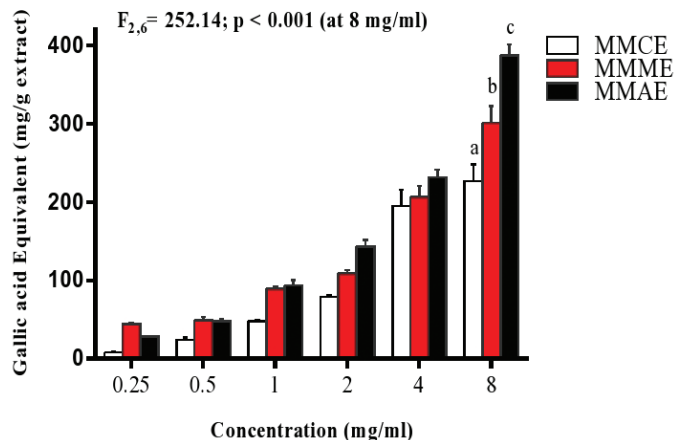
The present study revealed the presence of various naturally occurring compounds such as alkaloids, cardiac glycosides, saponins, steroids, tannins, and terpenoids in *M. macrophylla* extracts (Table 1). However, different solvent extracts of *M. macrophylla* contain different phytochemicals. These compounds, belonging to the polyphenolic group, have been reported to exhibited several pharmaceutical potentials. For example, tannins as anti-oxidants and anti-inflammatory (Dolara *et al.*, 2005), alkaloids as anti-hypertensive and anti-malarial (Wink *et al.*, 1998), terpenoids as anti-ulcer and anti-microbial (Cowan, 1999; Dudareva *et al.*, 2004), and saponins as anti-microbial and anti-inflammatory (Gangwar *et al.*, 2014; Lacaille-Dubois and Wagner, 2000).

### Total phenolic and flavonoid contents

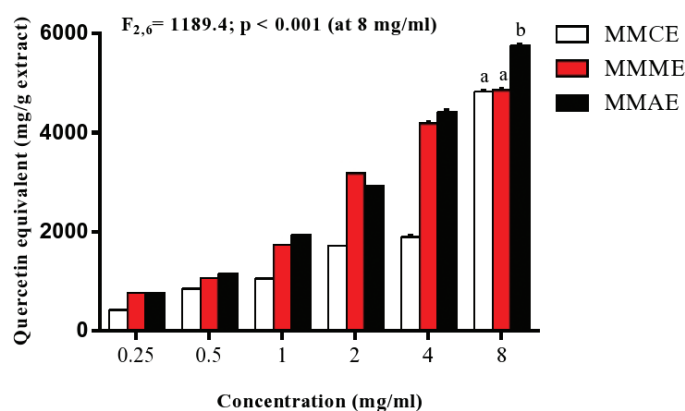
The total phenolic content of *M. macrophylla* extracts increased in a concentration-dependent manner (Fig. 1). The total phenolic content showed significant variation between different extracts of *M. macrophylla* ( $F_{2,6} = 252.14$ ;  $p < 0.001$ ). MMAE has the highest ( $p < 0.001$ ) total phenolic content ( $387.61 \pm 14.10$  mg GAE/g) followed by MMME ( $301.29 \pm 21.73$  mg GAE/g) and MMCE ( $226.9 \pm 21.04$  mg GAE/g). Similarly, dose-dependent increase in the total flavonoid content was observed among the various extracts of *M. macrophylla* (Fig. 2). At 8 mg/ml, there was a significant variation ( $F_{2,6} = 1,189.4$ ;  $p < 0.001$ ) in the flavonoid contents among *M. macrophylla* extracts. MMAE has the highest flavonoid content ( $5,761.65 \pm 38.5$  mg quercetin equivalent/g) followed by MMME ( $4,864.68 \pm 36.75$  mg quercetin equivalent/g) and MMCE ( $4,830.09 \pm 32.80$  mg quercetin equivalent/g). The present study indicated the presence of significant amounts of flavonoid and phenolic compounds in

**Table 1.** Preliminary phytochemical screening of different extracts of *M. macrophylla*. (“+” indicates presence of phytochemical and “-” indicates absence of phytochemical).

Phytochemicals	Reagent	Colour Indication	MMCE	MMME	MMAE
Alkaloids	Dragendorff's reagent	Reddish brown precipitate	-	+	+
Cardiac glycosides	Glacial acetic acid				
	Ferric chloride Sulphuric acid	Brown ring	-	+	+
Saponins	Olive oil	Whitish emulsion	-	+	+
Steroids	Sulphuric acid	Red colour	-	+	-
Tannins	Ferric chloride	Brownish green or blue-black	+	-	+
Terpenoids	Sulfuric acid	Reddish Brown	-	+	-
Phlobatannins	Hydrochloric acid	Red precipitate	-	-	-



**Figure 1.** Total phenolic content of various extracts of *M. macrophylla* determined as GAE. Values are expressed as mean  $\pm$  standard error mean (SEM),  $n = 3$ . Different letters indicate significant variation.



**Figure 2.** Total flavonoid content of various extracts of *M. macrophylla* determined as quercetin equivalent. Values are expressed as mean  $\pm$  SEM,  $n = 3$ . Different letters indicate significant variation.

*M. macrophylla* extracts. The phenolic compounds have been reported to have anti-oxidants property due to their conjugated ring structures and the presence of hydroxyl groups. Their scavenging activity is mostly achieved by hydrogenation and can even stabilize free radicals by complexing with oxidizing species (Amic *et al.*, 2003; Diplock, 1997). Compounds containing phenols have been reported to exhibit cardio-protective, hepato-protective, anti-allergenic, anti-inflammatory, anti-microbial, anti-thrombotic, anti-atherogenic, and vaso-dilatory effects (Alpinar *et al.*, 2009; Middleton *et al.*, 2000; Siama *et al.*, 2018). Similarly, flavonoids are having antioxidants, free radical scavenging, anti-inflammatory, metal chelating, and lipid peroxidation inhibitory properties due to their unique structural conformations (Cook and Samman, 1996). Due to their scavenging or chelating activities, flavonoids have been reported to serve as health-promoting compound (Havsteen, 1983) and protect humans against several diseases like atherogenesis, carcinogenesis, hepatotoxicity, and thrombosis (Tiwari, 2001). Flavonoids such as Aureusidin-4-glucoside, Aureusidin-6-glucoside, Aureusidin-4,6-diglucoside, and Cernuoside have been isolated from *M. hirsutissima* (Harborne and Girija, 1983). Quercetin and hypenins were also reported to occur in *M. frondosa* (Lakshmi *et al.*, 1985).

The root bark of *M. macrophylla* has been reported to contain triterpenoid glycosides including 3-O-β-D-glucopyranosyl-28-O-α-L-rhamnopyranosyl-16α-hydroxy-23-deoxy-protobassic acid, 28-O-β-D-glucopyranosyl-16α-hydroxy-23-deoxyprotobassic acid, 3-O-β-D-glucopyranosyl-28-O-α-L-rhamnopyranosyl-16α-hydroxyprotobassic acid, 3-O-acetyloleanolic acid, and 3-O-acetyldaturadiol. The triterpenoid glycosides isolated from *M. macrophylla* were reported to exhibit inhibitory action against *Porphyromonas gingivalis* (Kim *et al.*, 1999), *Salmonella paratyphi*, and *Aspergillus niger* (Chowdhury *et al.*, 2013).

### **In vitro free radical scavenging activities of *M. macrophylla***

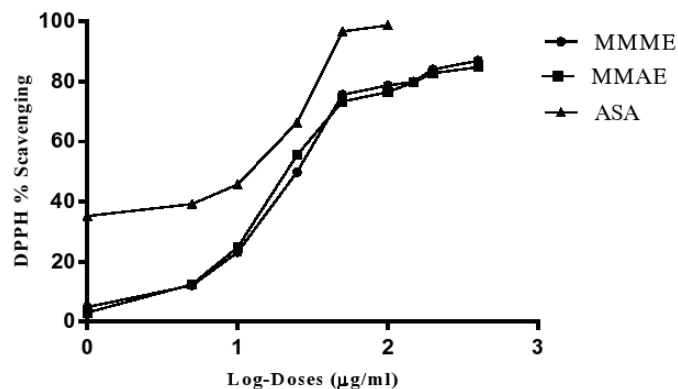
#### *DPPH radical scavenging activity*

The methanolic and aqueous extracts of *M. macrophylla* showed a dose-dependent increase in DPPH radicals scavenging activity as indicated by the discoloration of DPPH. Maximum scavenging activity for both methanolic and aqueous extracts was observed at a concentration of 400 µg/ml. Log-doses of various extracts of *M. macrophylla* and standard ASA were plotted against DPPH inhibition (%) for the calculation of IC<sub>50</sub> (Fig. 3). The aqueous extract (IC<sub>50</sub>: 25.92 ± 0.33 µg/ml) and methanolic extract (IC<sub>50</sub>: 26.43 ± 0.55 µg/ml) were found to have similar DPPH radical scavenging activity (Fig. 4), whereas the IC<sub>50</sub> of chloroform extract could not be determined within the given concentration.

The antioxidant property of natural compounds such as plant extracts can be evaluated through their ability to reduced methanolic DPPH solution to non-radical form DPPH-H. The antioxidative potential of *M. macrophylla* extracts was determined by their ability to scavenge DPPH radicals, and *M. macrophylla* extracts were found to be active radical scavengers. Compounds such as polyhydroxyl aromatic compounds, cysteine, tocopherol, glutathione, and ASA have been known to have the ability to reduce DPPH by hydrogenation (Blois, 1958; Moon *et al.*, 2010). Among the genus *Mussaenda*, *M. frondosa* (Siju *et al.*, 2010), *M. glabrata* (Menon and Sasikumar, 2011), and *M. roxburghii* (Islam *et al.*, 2015) have been reported to effectively scavenge the DPPH radicals.

#### *Scavenging activity of superoxide radical*

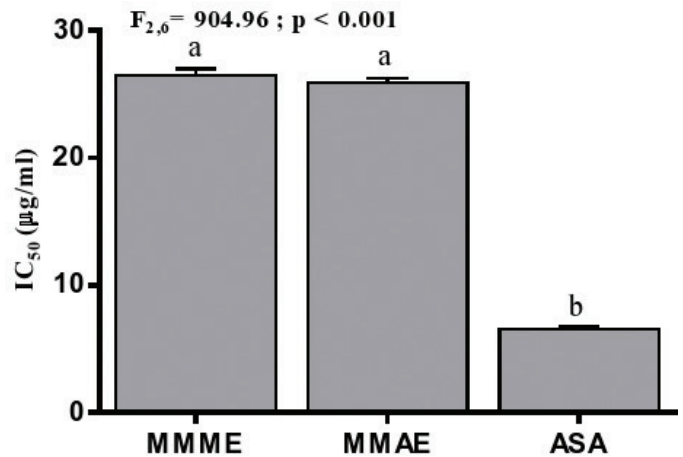
Various extracts of *M. macrophylla* showed a dose-dependent increase in inhibition of superoxide radical (O<sub>2</sub><sup>•-</sup>) generation. Maximum O<sub>2</sub><sup>•-</sup> scavenging activity was recorded at



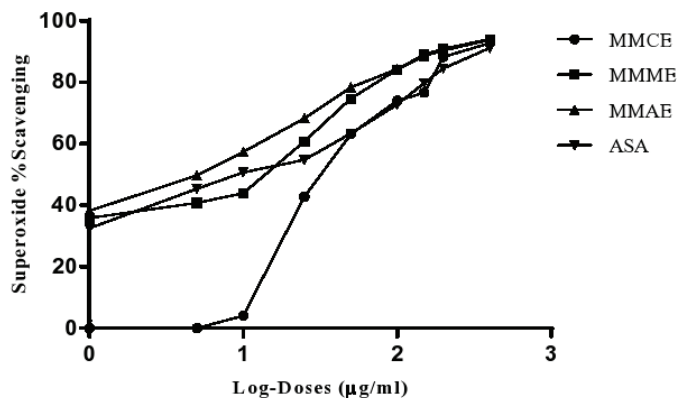
**Figure 3.** Plot of log-doses of various extracts of *M. macrophylla* and standard ASA against DPPH inhibition (%) for the calculation of IC<sub>50</sub>.

400 µg/ml in all the extracts of *M. macrophylla*. Figure 5 represents the plot of Log-doses of various extracts of *M. macrophylla* and standard ASA against O<sub>2</sub><sup>•-</sup> scavenging activity for the calculation of IC<sub>50</sub>. Aqueous extract possessed the highest scavenging activity (IC<sub>50</sub>: 4.12 ± 0.94 µg/ml) followed by methanolic extract (IC<sub>50</sub>: 7.83 ± 1.2 µg/ml) and chloroform extract (IC<sub>50</sub>: 40.24 ± 3.5 µg/ml). The O<sub>2</sub><sup>•-</sup> scavenging activity of MMAE was even better than the ASA (IC<sub>50</sub>: 8.65 ± 1.6 µg/ml) (Fig. 6).

Superoxide anion radical, a highly reactive free radical, leads to cellular damage by initiating lipid oxidation indirectly (Aruoma and Halliwell, 1987). Superoxide (O<sub>2</sub><sup>•-</sup>) radical can break down to form stronger reactive oxygen species (ROS) including hydroxyl radicals and singlet oxygen. Thus, scavenging of O<sub>2</sub><sup>•-</sup> could result in inhibition of other ROS generation and protect the cells from oxidative damage. The present study suggest that O<sub>2</sub><sup>•-</sup> radical scavenging activity of *M. macrophylla* extracts increased with increases in concentration and the aqueous extract of *M. macrophylla* showed higher scavenging potential than the standard ASA. The O<sub>2</sub><sup>•-</sup> scavenging activity of *M. macrophylla* extracts could be attributed to their flavonoids contents (Robak and Gryglewski, 1988).



**Figure 4.** IC<sub>50</sub> (µg/ml) for DPPH of various extracts of *M. macrophylla* and standard ASA. Values are expressed as mean ± SEM, n = 3. Different letters indicate significant variation.



**Figure 5.** Plot of log-doses of various extracts of *M. macrophylla* and standard ASA against superoxide radical inhibition (%) for the calculation of IC<sub>50</sub>.

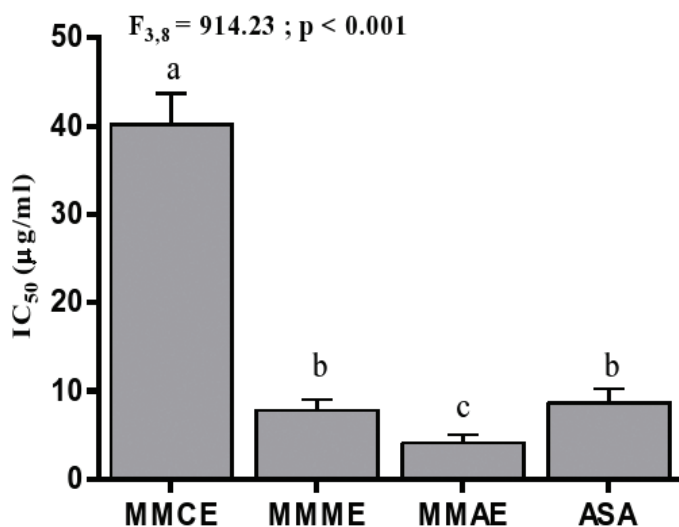
### ABTS radical scavenging activity

ABTS<sup>•+</sup> radical scavenging property of *M. macrophylla* extracts increased in a concentration-dependent manner as indicated by dis-coloration of the ABTS<sup>•+</sup>. Maximum scavenging activity was recorded at 200 µg/ml for all the extracts of *M. macrophylla*. Log-doses of various extracts of *M. macrophylla* and standard ASA were plotted against ABTS<sup>•+</sup> inhibition (%) for the calculation of IC<sub>50</sub> (Fig. 7). MMAE possessed the highest scavenging activity (lowest IC<sub>50</sub>: 17.20 ± 1.5 µg/ml) followed by MMME (IC<sub>50</sub>: 25.95 ± 1.8 µg/ml) and MMCE (IC<sub>50</sub>: 197.16 ± 5.5 µg/ml). The aqueous and methanolic extracts of *M. macrophylla* were found to be more effective in scavenging ABTS<sup>•+</sup> radical than the standard ASA (IC<sub>50</sub>: 39.70 ± 1.2 µg/ml) (Fig. 8). There was significant positive correlation between phenol ( $r^2 = 1.00$ ;  $p < 0.001$ ) and flavonoid ( $r^2 = 0.99$ ;  $p < 0.001$ ) contents of *M. macrophylla* extracts and their free radicals (DPPH, O<sub>2</sub><sup>•-</sup> and ABTS) scavenging activities.

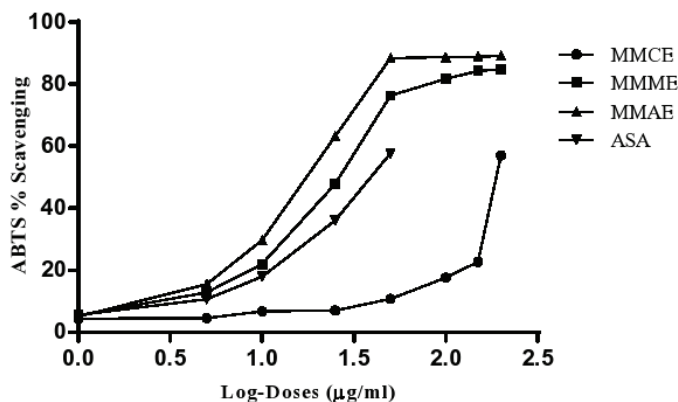
The antioxidative activity of various extracts of *M. macrophylla* was also determined by measuring their ability to convert the blue colored ABTS<sup>•+</sup>, which is formed by the interaction of ABTS and potassium ferricyanide, to ABTS. The effectiveness of this conversion depends on a number of characteristics such as an abundance of aromatic rings, types of hydroxyl group's substitution, and the molecular weight of phenolic compounds (Hagerman *et al.*, 1998). The study demonstrates *M. macrophylla* extracts as potent antioxidants and their ABTS<sup>•+</sup> scavenging activity could be due to their phenolic contents.

### Reducing power

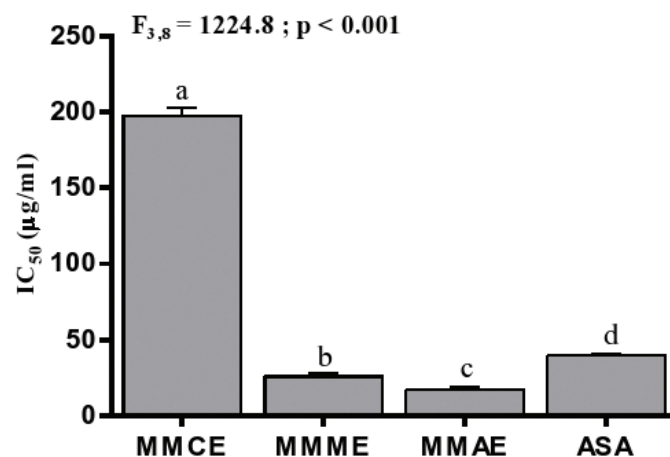
The reducing power of various extracts of *M. macrophylla* was determined by measuring the transformation of Fe<sup>3+</sup> to Fe<sup>2+</sup>. The reducing activity of *M. macrophylla* extracts increased in a dose-dependent manner (Fig. 9). At 400 µg/ml, the highest reducing activity was recorded for MMME (1.098 ± 0.0003) followed by MMAE (0.731 ± 0.002) and MMCE (0.527 ± 0.0008). The reducing activity of MMME was higher than the standard ASA (0.87 ± 0.02).



**Figure 6.** IC<sub>50</sub> (µg/ml) for superoxide radical of various extracts of *M. macrophylla* and standard ASA. Values are expressed as mean ± SEM,  $n = 3$ . Different letters indicate significant variation.



**Figure 7.** Plot of log-doses of various extracts of *M. macrophylla* and standard ASA against ABTS inhibition (%) for the calculation of IC<sub>50</sub>.



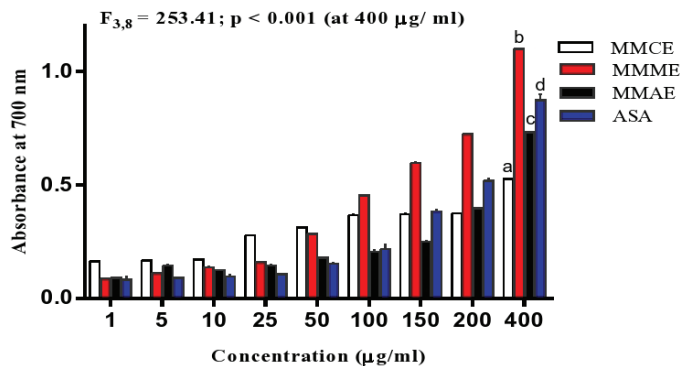
**Figure 8.** IC<sub>50</sub> (µg/ml) for ABTS of various extracts of *M. macrophylla* and standard ASA. Values are expressed as mean ± SEM,  $n = 3$ . Different letters indicate significant variation.

The reducing power of compounds serves as an indicator of their antioxidant property (Meir *et al.*, 1995). The higher absorbance at higher concentrations showed the high reducing power potential of the extracts. The reducing activity of MMME being higher than the standard ASA indicates the strong redox potential of the extract. The graded increase in percentage inhibition as the concentration of the plant extracts increases indicates that percentage scavenging effectiveness strongly depends on the extracts concentrations.

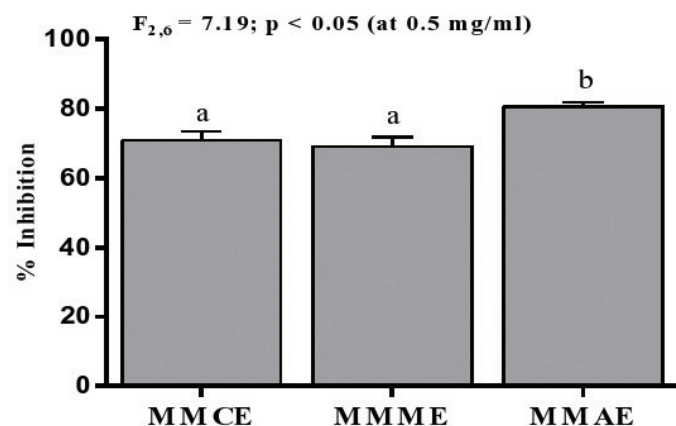
### Ex vivo antioxidant assay

#### Anti-hemolytic activity and lipid peroxidation inhibition

The anti-hemolytic activity was determined using 0.5 mg/ml of various extracts of *M. macrophylla*. There was a significant variation in the anti-hemolytic activity of different extracts of *M. macrophylla* ( $F_{2,6} = 7.19$ ;  $p < 0.05$ ). MMAE showed the highest ( $p < 0.05$ ) inhibitory activity against erythrocyte hemolysis with an inhibition rate of 80.53% followed by MMCE (70.91%) and MMME (69.17%) (Fig. 10). The lipid peroxidation inhibition potential of *M. macrophylla* extracts was also estimated in mice liver homogenate. Significant variation was observed



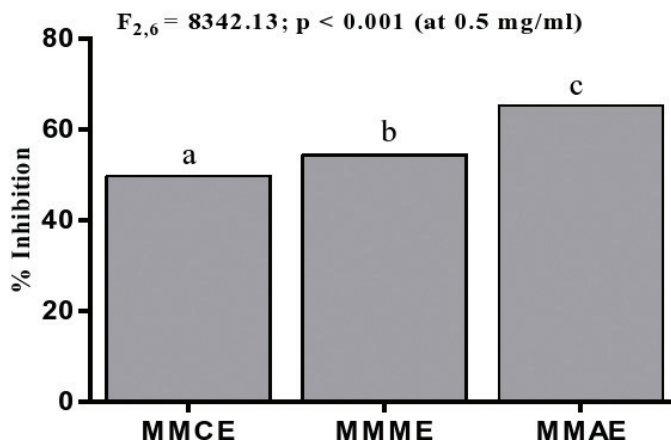
**Figure 9.** Reducing power of various extracts of *M. macrophylla* and standard ASA. Values are expressed as mean  $\pm$  SEM,  $n = 3$ . Different letters indicate significant variation.



**Figure 10.** Anti-hemolytic activity of various extracts of *M. macrophylla*. Values are expressed as mean  $\pm$  SEM,  $n = 3$ . Different letters indicate significant variation.

among *M. macrophylla* extracts in their inhibitory activity against lipid peroxidation ( $F_{2,6} = 8,342.13$ ;  $p < 0.001$ ). Highest inhibitory activity was recorded for MMAE with an inhibition rate of 65.33% followed by MMME (54.38%) and MMCE (Fig. 11).

Hemolysis occurs due to membrane damage caused by the activity of free radicals on erythrocytes that are the major target of free radicals (Ebrahimzadeh *et al.*, 2009; Wang and Yao, 2005). Peroxidation of lipid moieties such as polyunsaturated fatty acids by a chain reaction of free radicals can lead to membrane damage (Klauning *et al.*, 1998). Lipid peroxidation in mice liver homogenate was induced by  $\text{FeCl}_2\text{-H}_2\text{O}_2$ . The formation of malonaldehyde is used as an indicator of lipid peroxidation, and subsequently oxidative stress. Various extracts of *M. macrophylla* showed significant anti-hemolytic and lipid peroxidation inhibition potential which could be due to the presence of numerous amounts of phenols and flavonoids. Certain phenolic compounds have been reported to partition in the cell membrane, hindering the diffusion of free radicals and consequently decreased the chain reaction of free radicals (Singh and Rajini, 2008). Flavonoids have been reported to inhibit lipid peroxidation in the erythrocytes membrane and improved their integrity against lyses by binding to the membrane (Chaudhuri *et al.*, 2007).



**Figure 11.** The lipid peroxidation inhibitory activity of various extracts of *M. macrophylla*. Values are expressed as mean  $\pm$  SEM,  $n = 3$ . Different letters indicate significant variation.

## CONCLUSION

The present study indicated that *M. macrophylla* extracts possess strong antiradical and antioxidant properties. The presence of significant amounts of flavonoid and phenolic compounds in the plant might be the cause of antioxidant activities as a significant positive correlation was observed between phytochemical contents and the free radicals scavenging activity. Although the mechanism is still unclear, the protective effects of *M. macrophylla* extracts against hemolysis and lipid peroxidation may be attributed due to the presence of various polyphenolic compounds. However, the exact phytochemical constituent responsible for radical scavenging activity and antioxidative potential of the plant needs to be investigated further.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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