

## RAPID *IN VITRO* CALLOGENESIS AND PHYTOCHEMICAL SCREENING OF LEAF, STEM AND LEAF CALLUS OF *MUSSAENDA FRONDOSA* LINN.: A MEDICINAL PLANT

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### **ABSTRACT**

**Objective:** To standardize the protocol for rapid callogenesis in *Mussaenda frondosa* L. using leaf explants. Qualitative and quantitative phytochemical analysis of leaf, stem, and callus cultures.

**Methods:** The leaf explants were inoculated onto Murashige and Skoog medium supplemented with varying concentrations of growth regulators such as 2, 4-D, NAA, benzylaminopurine, Kn for the induction of callus. Qualitative and quantitative analysis of total phenol, flavonoid and alkaloid contents of leaf, stem, and callus were tested by standard methods. The antioxidant activities were investigated using 1, 1- diphenyl-2-picryl hydrazyl radical scavenging method and reducing power assay. The anti-inflammatory activity was evaluated by membrane stabilizing activity.

**Results:** Pale green, healthy, friable, and fast growing callus were obtained on the medium enriched with NAA (2 mg/l)+Kn (4 mg/l). Quantitative determination showed the highest concentration of total phenolics in the methanolic extract of *in vitro* grown callus ( $10\pm1.1$  mg of GA/g of extract), flavonoids in methanolic stem extract ( $137\pm1.6$  mg of quercetin/g of extract) and alkaloids in methanolic extract of leaf ( $118.3\pm1.5$  mg/10 g of extract). The methanolic leaf extract exhibited the highest free radical scavenging activity with an inhibitory concentration 50% ( $IC_{50}$ ) value of  $40.6\pm10.06$   $\mu$ g/ml. The highest membrane stabilizing activity was shown by chloroform extract of the leaf (66.02%).

**Conclusion:** This preliminary phytochemical and pharmacological analysis may form the basis for drug development in future using callus cultures of *M. frondosa*.

**Keywords:** *Mussaenda frondosa*, Antioxidant, Anti-inflammatory, *In vitro* culture, Phenolics, Flavonoids, Alkaloids.

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### **INTRODUCTION**

Plant products contain a large number of pharmacologically active compounds which are being used in traditional medicine since Vedic era. These can be derived from bark, leaves, flowers, roots, fruits, and seeds [1]. Synthetic drugs and antibiotics are found to have many adverse effects and sometimes fatal. Hence, medicinal plants from natural flora are being explored for the synthesis of commercial drugs [2]. Demand for plant products has been increased with increasing population causing depletion of medicinal plants in the natural habitat. The plant tissue culture techniques proved to be an alternative for the production of valuable phytochemicals [3]. Considering the potential and promises of plant cell/tissue culture technology, efforts have been made to improve the productivity of pharmacologically important plant species [4].

Plant cell cultures offer an alternative source to the whole plant for the production of high-value secondary metabolites [5]. Researchers have endeavored to utilize plant cell biosynthetic capabilities for obtaining useful products and for studying the metabolism [6]. A large number of metabolites such as alkaloids, saponins, cardenolides, anthraquinones, polyphenols, and terpenes have been reported from *in vitro* cultures for e.g. flavonoids and stilbenes [7], sterols [8], cardenolides [9], and phenolic acids [10]. Several strategies have been adopted for the enhancement of *in vitro* bioactive metabolite production; one such method is, use of growth regulators which is often a crucial factor in secondary product accumulation [11].

*Mussaenda frondosa* syn. *Mussaenda glabrata* (Family: Rubiaceae) is a rambling shrub seen in the Western Ghats, Andamans, Konkan, Malabar

and Tirunelveli hills. The foliage is lighter green, and the terminal flower clusters have orange to yellow, tubular corollas with a single white enlarged calyx lobe. This species is often grown in clumps [12]. Traditionally, leaves are used in the treatment of jaundice, asthma, hyperacidity, fever, ulcers, leprosy, wound, swells [13] and as an antimicrobial agent [14]. This plant has been investigated by several workers for diuretic [15], hepatoprotective [16], hypolipidemic activities [17], radical scavenging [18] and to treat fever, asthma, and cough. Siju *et al.* [19] have reported the antihelmintic activity of the ethyl alcohol and aqueous extract of the plant. Flowers are used in detoxifying mushroom poisons and terminate an early pregnancy [20].

Iridoids, flavonoids, and triterpenes are the important bioactive compounds distributed in *Mussaenda* species. Phytochemicals such as astragalin, isoquercetin, and kaempferol-3-o-beta rutinoside were isolated from leaves [21]. A new compound sanzhilactone along with mussaenside, barlerin, lupeol and beta-d-glucose has been obtained from the stem [22]. Quercetin (QE), rutin, hyperin, ferulic acid, synaptic acid, beta-sitosterol, saponins have also been isolated [23].

Reports are available on the *in vitro* callus culture of *Mussaenda erythrophylla* cvs. [24]. As per the available literature, there are no reports available on the *in vitro* callogenesis of *M. frondosa*. Therefore, in this work, the effect of phytohormone combinations on the *in vitro* callus production of *M. frondosa* was investigated, and *in vitro* experiment was conducted to determine antioxidant and anti-inflammatory effect of leaf, stem, and callus extracts of *M. frondosa*.

## METHODS

### Plant material and surface sterilization

The leaves of *M. frondosa* were collected from natural forests of Dakshina Kannada district, Karnataka, India. The collected materials were identified following the flora of Udupi and Dakshina Kannada [25]. A voucher specimen (MU/AB/DJM - 02) has been deposited in herbarium collection at Department of Applied Botany, Mangalore University.

The mature, young and the youngest leaf samples were collected to find out the suitability of explants for *in vitro* callus induction. The fresh and healthy leaves were washed thoroughly under running tap water for 1 hr to remove the surface debris. The leaves were surface sterilized by 1% bavistin for 1 hr followed by treating with 70% alcohol for 1 minute and 0.1% mercuric chloride for 1 minute after each step, the explants were rinsed thoroughly with sterile distilled water. Later, the explants were cut into 1 cm<sup>2</sup> and inoculated onto Murashige and Skoog (MS) medium [26] at 25±2°C and a photoperiod of 16 hrs light and 8 hrs dark for 30 days. Observations like a percentage (%) of browning, fungal contamination; bacterial contamination and explants survival rate were made until 3 weeks of inoculation.

### Callus induction

The sterilized young leaf explants were inoculated onto MS medium fortified with different concentrations of growth regulators such as 2,4-dichlorophenoxyacetic acid (2, 4-D), -naphthaleneacetic acid (NAA), benzylaminopurine (BAP), and Kinetin (KN) for the induction of callus (Table 1). All the cultures were incubated at 25±2°C with 16 hrs of photoperiod and 40.0±3.0 mM m<sup>-2</sup> s<sup>-1</sup> light intensity. Twenty explants were inoculated to each combination of growth regulators with one explant per test tube and replicated 3 times. The explants were observed at regular intervals for various details such as the development, color, and texture of callus. The calli were subcultured at 3 weeks of intervals.

### Preparation of the plant and callus crude extract

The dried leaf, stem samples of *M. frondosa* were coarsely powdered and were extracted with methanol, chloroform, dichloromethane (DCM) and water by Soxhlet extraction for 36 hrs. The extracts obtained were evaporated to dryness and were stored in the fridge until further use.

Callus was dried in oven at 50°C till constant weight was achieved. It was then coarsely powdered using mortar and pestle and extracted in methanol, chloroform, DCM and water in Soxhlet apparatus for 36 hrs. The extracts were concentrated till dry and were stored in the fridge for future analysis.

### Qualitative and quantitative analysis of total phenol, flavonoid, and alkaloid contents

The leaf, stem, and *in vitro* grown callus extracts were subjected to preliminary phytochemical screening to determine the presence of secondary metabolites such as alkaloids, flavonoids, terpenoids, phenols, tannins, and glycosides by standard methods [27,28]. Total phenolic, flavonoid, and alkaloid contents in the crude extract were determined as per Harborne [27], Ikram *et al.* [29], and Sreevidya and Mehrotra [30], respectively. Free radical scavenging activities of the extracts were determined using 1, 1-diphenyl-2-picryl hydrazyl radical (DPPH) as per Zishen *et al.* [31] and the reducing power by the method of Oyaizu [32]. Anti-inflammatory activity was determined by membrane stabilizing activity as per Shinde *et al.* [33].

### Solvents and chemicals

All the chemicals used were of analytical grade procured from Sigma-Aldrich. The growth regulators were obtained from Hi-Media.

### Statistical analysis

Experiments were repeated thrice (n=3). Data were analyzed by SPSS statistical package software version 20 with Duncan's multiple range test grouping. Statistical differences were considered significant at \*p<0.05.

**Table 1: Effects of PGRs on callus induction and callus growth of leaf segments of *M. frondosa***

PGRs	Concentration of PGRs (mg/l)	% of callus induction	Response intensity	Texture of callus
Control	-	0	-	-
MS+2, 4-D	0.5	50	+	Friable
	1	68	++	
	2	75	++	
	3	78	++	
MS+NAA	0.5	50	+	Friable
	1	75	++	
	2	90	++++	
	3	90	++++	
MS+BAP	0.5	80	+	Friable
	1	70	++	
	2	60	++	
	3	54	++	
MS+Kn	0.1	20	+	Friable
	0.5	70	++	
	1	50	+	
	1.5	55	+	
MS+2,4 D+Kn	0.5+0.1	20	+	Friable
	1+0.5	45	+	
	2+1	65	++	
	3+2	80	++	
MS+2,4 D+BAP	0.5+0.1	60	++	Friable
	1+0.5	80	+++	
	2+1	90	++++	
	3+2	90	++++	
MS+NAA+BAP	0.1+1	50	+	Friable
	0.5+2	60	++	
	1+3	90	++++	
	2+4	90	++++	
MS+NAA+Kn	0.1+1	60	++	Friable
	0.5+2	80	+++	
	1+3	90	++++	
	2+4	90	++++	

+: Poor response, ++: Moderate response, +++: Good response, ++++: Very good response, PGRs: Plant growth regulators, *M. frondosa*: *Mussaenda frondosa*, MS: Murashige and Skoog, NAA: 1-naphthaleneacetic acid

## RESULTS AND DISCUSSION

### Callus induction and establishment

Young leaf segments were transferred to different media for induction of callus expanded on all media after 4-5 days of inoculation. Calli were developed from the cut surfaces of the leaf, midrib regions and also from the surface of explants. All the combinations of growth hormones showed the development of callus in varying percentage. The maximum rate of callus proliferation was obtained on MS medium supplemented with different combinations of growth hormones such as NAA (2 mg/L)+BAP (4 mg/L) (Fig. 1a), 2,4-D (2 mg/L)+BAP (1 mg/L) (Fig. 1b) 2 and 3 mg/L NAA (Fig. 1c), NAA (2 mg/L) + Kn (4 mg/L) (Fig. 1d), 2,4-D (3 mg/L)+BAP (2 mg/L), NAA (1 mg/L)+BAP (3 mg/L), and NAA (1 mg/L)+Kn (3 mg/L) (Table 1). Compact yellow callus produced on the MS media supplemented with different concentrations of kinetin (0.1-1.5 mg/L) showing a poor response. NAA, at all concentrations, produced friable callus which turned brown after subculturing (Fig. 1e). Callus developed on MS media with 2,4-D+BAP, NAA+BAP, and NAA (1 mg/L)+Kn (3 mg/L) was slow growing, turned brown and failed to proliferate after subculturing after 3-4 weeks. Since NAA (2 mg/L)+ Kn (4 mg/L) was found to be the best for callus induction with pale green, healthy, friable, and fast growing this was selected for further experiments.

MS medium supplemented with 1.0 mg/L BA and 2.0 mg/L indole-3-acetic acid (IAA) was found to be best suitable for callus proliferation in *Mussaenda erythrophylla* cv [24]. Combination of 2, 4-D (1 mg/L)

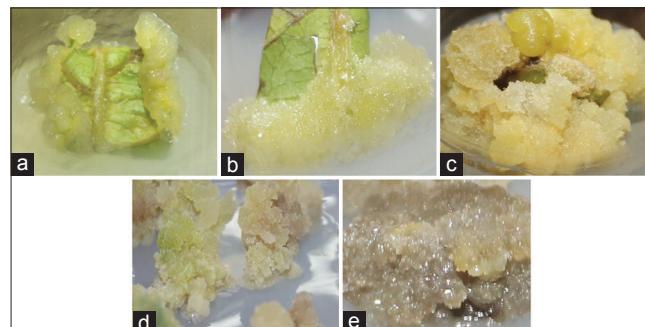
with BA (0.5 mg/l) for callus induction was reported in *Plumbago zeylanica* [34]. Bhagya and Chandrashekhar [35], proved MS medium with NAA+BAP (1+0.1 mg/L) gave the best results for callus induction in *Justicia gendarussa* Burm. f.

Preliminary phytochemical analysis of *M. frondosa* leaf, stem, and callus extract shown in Table 2. Phenolics, flavonoids, alkaloids and glycosides detected in leaf, stem and callus extracts. Tannins and triterpenoids were found to be absent in leaf. Chloroform extract of stem and callus indicated the presence of all the phytochemicals tested except for triterpenoids, steroids, and resins. Chloroform extract of leaf, stem, and callus showed a maximum number of phytochemicals tested. Similar results were obtained in the phytochemical screening of the *Tecoma stans* leaves extracted in different solvents [36], whereas Thalwal *et al.* found methanol to be the better solvent for the extraction of phytochemicals in *Amaranthus spinosus* [37]. These phytochemicals are responsible for various biological activities. The importance of these metabolites in treating various diseases reported by Kubmarawa *et al.* [38] and Mensah *et al.* [39].

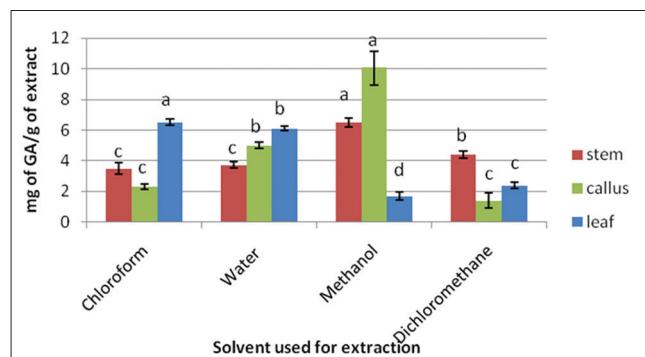
Varying concentrations of phenol, flavonoid and alkaloid contents were detected in leaf, stem, and callus of *M. frondosa* extracts. The highest concentration of total phenolics was present in the methanolic extract of *in vitro* grown callus ( $10 \pm 1.1$  mg of GA/g of extract) (Fig. 2). The phenolic content in the leaf and stem extracts ranged between  $1.7 \pm 0.2$  and  $6.5 \pm 0.2$  mg of GA/g of extract and  $3.5 \pm 0.3$  to  $6.5 \pm 0.3$  mg of GA/g of extract, respectively whereas, in callus extract it was found in the range between  $1.4 \pm 0.5$  and  $10 \pm 1.1$  mg of GA/g of extract. Similar results were reported in *J. gendarussa* Burm. f [35], *Alangium salviifolium* [40] and *Cyclea peltata* [41]. The effect of growth regulators on the biosynthesis of phenolic compounds was studied in hairy roots of *Panax ginseng* by Jeong *et al.* [42]. Taj Al-Deen [43] recorded low value of total phenolic content 128.67 mg/g of dry weight of an alcoholic extract of callus derived from hypocotyls of *Trigonella foenum-graecum* compared to plant parts. The solvent system plays an important role in the solubility of phytochemical components in the crude extracts [44].

This study showed maximum flavonoid content in the stem extracts when compared to leaf and callus extract (Fig. 3). Methanolic stem extract possesses significantly the highest amount of flavonoid content ( $137 \pm 1.6$  mg of QE/g of extract). Total flavonoid content in the leaf ranged between  $35.3 \pm 1.5$  and  $53.8 \pm 1.7$  mg of QE/g of extract. In callus extract, the total flavonoid ranged between  $12.4 \pm 1.4$  and  $76 \pm 1$  mg of QE/g of extract, with a highest in the methanolic extract. Umesh [45], spectrophotometrically determined total flavonoids in methanolic extract of *Decalepis hamiltonii* (Wight and Arn) in roots and callus as  $180.5 \pm 63.36$   $\mu$ g (QE)/mg and  $130.6 \pm 3.571$   $\mu$ g (QE)/mg of extract, respectively. Rami and Patel [46] found maximum flavonoid content of  $1.025 \pm 0.002$  mg/g in methanolic extract of *in vitro* developed root when compared to wild plant and other *in vitro* plant parts.

It is well known that the concentrations of phenolic content are usually higher than the concentrations of flavonoid in most cases, but a reverse trend was observed in this study. It can be explained by the fact that differences in the polarity of the phytochemicals toward different solvents used in the experiment. Furthermore, it is well known that the Folin-Ciocalteu method is a rapid and widely used assay to detect the total phenolic content, but different phenolic compounds have different responses in the Folin-Ciocalteu method [47].



**Fig. 1: *In vitro* callus induction from leaf explants of *Mussaenda frondosa*. Callus initiation from the leaf explant grown on Murashige and Skoog (MS) medium supplemented with (a) NAA (2 mg/L) + benzylaminopurine (BAP) (4 mg/L). (b) 2, 4-D (2 mg/L)+BAP (1 mg/L). (c) NAA (2 mg/L). (d) Subcultured callus on MS medium supplemented with NAA (2 mg/L) + Kn (4 mg/L). (e) Callus turned brown after subculturing to MS media supplemented with NAA (3 mg/L)**



**Fig. 2: Comparative analysis of total phenols present in leaf, stem and callus of *Mussaenda frondosa*. Values are mean $\pm$ standard deviation each of three replicates (n=3). Different alphabets indicate significant difference at \*p<0.05**

**Table 2: Preliminary phytochemical screening of *M. frondosa* leaves, stem, and callus**

Phytochemicals	<i>M. frondosa</i>				Leaf				Stem				Callus			
					CHL	WAT	MET	DCM	CHL	WAT	MET	DCM	CHL	WAT	MET	DCM
Extract																
Phenols	+	-	+	+	+	-	+	+	+	-	-	-	+	-	-	+
Flavonoids	+	+	+	+	+	+	+	+	+	-	-	-	+	-	+	+
Tannins	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-
Saponins	+	-	-	+	+	+	+	-	-	-	-	-	+	+	-	-
Alkaloids	+	-	+	+	+	-	-	+	+	-	-	-	+	-	-	+
Glycosides	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+
Triterpenoids	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Resins	+	-	+	+	-	-	-	-	-	-	-	-	+	-	-	+
Steroids	+	-	+	+	-	+	-	-	+	-	-	-	+	+	+	+

CHL: Chloroform, WAT: Water, MET: Methanol, DCM: Dichloromethane, (+): Present, (-): Absent, *M. frondosa*: *Mussaenda frondosa*

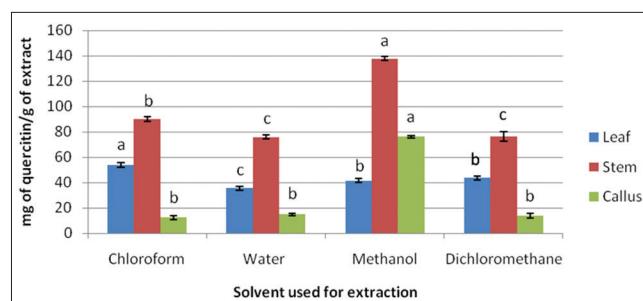
A significantly higher yield of  $118.3 \pm 1.5$  mg/10 g of alkaloid was observed in methanolic extract of the leaf (Fig. 4). Leaf extracts showed the alkaloid content ranging from  $118.3 \pm 1.5$  to  $21.6 \pm 1.3$  mg/10 g of extract. The alkaloid content in the callus extract is between  $104.5 \pm 1.5$  and  $98 \pm 1.2$  mg/10 g of extract, and in stem extract it is between  $106.4 \pm 1.5$  and  $60.4 \pm 1.8$  mg/10 g of extract. Similar results were obtained in *Leonurus heterophyllus* Sw. [48]. In contrast, enhanced accumulation of total alkaloids was observed *in vitro* grown callus of *Withania somnifera* [49].

#### Antioxidant activity

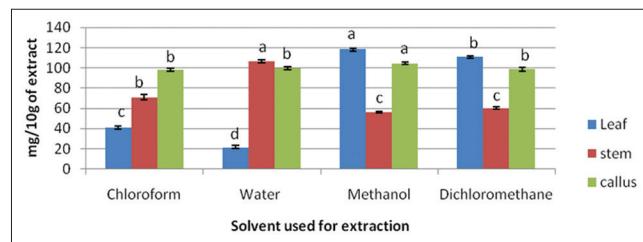
Methanolic extract of leaf and callus showed an on par DPPH reducing activity with an inhibitory concentration 50% ( $IC_{50}$ ) value of  $40.6 \pm 10.06$  µg/ml and  $52 \pm 11.35$  µg/ml, respectively (Fig. 5). Similar results were obtained in *D. hamiltonii* (Wight and Arn), in which the radicle scavenging activity was highest in root extract when compared to the *in vitro* grown callus with an  $IC_{50}$  values of  $20 \pm 1.54$  and  $148 \pm 4.39$  µg/ml, respectively. However, the  $IC_{50}$  value for stem extract was much higher compared to leaf and callus. The free radical scavenging activity of the standard ascorbic acid was found to be more than the extracts with an  $IC_{50}$  value of 15 µg/ml. In contrary, the highest DPPH scavenging activity was recorded in the *in vitro* grown callus than the plant parts by Babeet et al. [50] in *A. salviifolium* Linn. and Bhagya and Chandrashekhar [35] in *J. gendarussa* Burm. f.

The reducing power of a compound may serve as a significant reflection of antioxidant activity, as they protect the system by the damaging effect of reactive oxygen species like hydroxyl radical. The reducing ability of a compound depends on the presence of reductants [51], which have been exhibited antioxidative potential by breaking the free radical chain, donating a hydrogen atom [52].

The extracts of *M. frondosa* showed concentration-dependent reducing power. The highest reductive capacity was observed in the methanolic extracts of the samples tested. Higher absorbance value indicates a stronger reducing power of the samples (Fig. 6). Antioxidant compounds can donate electrons to reactive radicals, reducing them into more stable and unreactive species [53].



**Fig. 3: Comparative analysis of total flavonoids present in the stem, leaf and callus of *Mussaenda frondosa*. Values are mean±standard deviation each of three replicates (n=3). Different alphabets indicate significant difference at \*p<0.05**



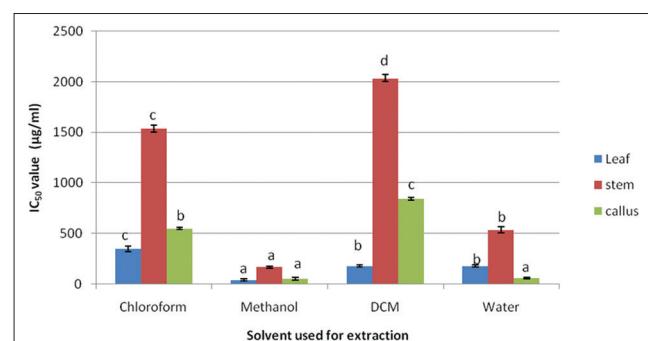
**Fig. 4: Comparative analysis of total alkaloids present in the stem, leaf and callus of *Mussaenda frondosa*. Values are mean±standard deviation each of three repeats (n=3). Different alphabets indicate significant difference at \*p<0.05**

#### Anti-inflammatory activity

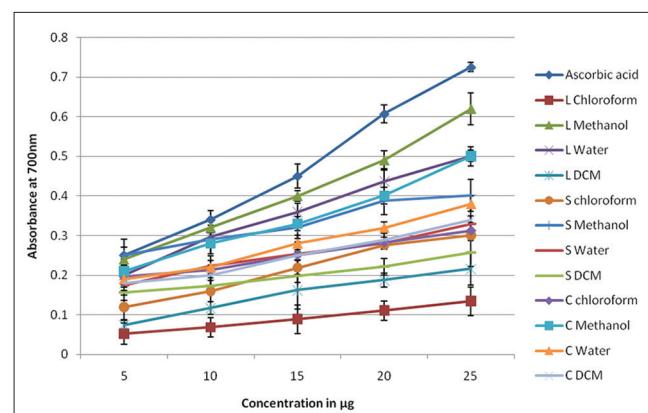
The effect of extracts of all the parts on the stabilization of human red blood cell (HRBC) membrane shown in Table 3. Chloroform extract of leaf, stem, and callus extracts showed 66.02%, 53.86%, and 65.91% HRBC membrane stabilization activity, respectively. Deepika and Vidya [54] reported a similar result where the maximum stabilization percentage was obtained in the leaf extracts of *Pluchea lanceolata* Oliver and Hiern (86.8% at 1000 µg/ml) as compared to stem, root, and callus samples. Deepika et al. [55] also reported the maximum stabilization percentage in leaves (88.8% at a dose of 1000 µg/ml) compared to stem and callus samples in *Coccucus hirsutus* (L.) Diels. Contrary results were reported by Taylor and Van Staden [56] in the plant *Eucomis autumnalis* (Mill.) Chitt. However, the extracts showed the lower membrane stabilization activity when compared to the standard drug diclofenac - sodium which showed 70.14% stabilization.

#### CONCLUSION

Medicinal plants form a large group of economically important plants that provide the necessary raw materials for indigenous pharmaceuticals. Results of the phytochemical evaluation of different extracts of *M. frondosa* revealed the presence of alkaloids, flavonoids, phenols, glycosides, saponins, and steroids. The pharmacological screening of leaf, stem, and callus extracts showed good antioxidant and anti-inflammatory activity. Hence, the callus cultures producing antioxidant and anti-inflammatory compounds might serve as model systems to investigate the regulation and production of these essential metabolites. The study will provide an efficient *in vitro* protocol for callus induction for the production



**Fig. 5: The inhibitory concentration 50% value of leaf, stem and *in vitro* grown callus of *Mussaenda frondosa*. Values are mean±standard deviation each of three replicates (n=3). Different alphabets indicate significant difference at \*p<0.05**



**Fig. 6: Reducing power assay of leaf, stem and callus of *Mussaenda frondosa*. L - Leaf, S - Stem, C - Callus, DCM - Dichloromethane. Values are mean±standard deviation each of three repeats (n=3)**

**Table 3: Effect of leaf, stem, and callus extracts of *M. frondosa* on anti-inflammatory activity by HRBC membrane stabilization**

Solvent	HRBC membrane stabilization (in % at 100 µg/L)		
	Leaf	Stem	Callus
Chlorform	66.02	53.86	65.91
Methanol	18.24	43.86	48.42
DCM	21.4	33.64	32.87
Water	54.47	12.87	40.64
Standard	70.14		
Diclofenac sodium			

HRBC: Human red blood cell, *M. frondosa*: *Mussaenda frondosa*,

DCM: Dichloromethane

of bioactive molecules. If appropriate strategies developed, there will be possibilities of making plant cell factories for the enhanced production of bioactive molecules, which may reduce the pressure on the natural plant resources.

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