

# Phytochemical and Pharmacological Profiling of *Morinda pubescence* Extract as Potential Agent for Alzheimer's and Diabetes Mellitus Dual Therapy

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## ABSTRACT

**Background:** Numerous epidemiological studies have shown that metabolic disturbances associated with the Diabetes Mellitus (DM) are recognized to be linked with brain atrophy as well as the pathological hallmarks of Alzheimer's Disease (AD). Therefore, development of multi-targeted agents found to be one of the best options to combat co-occurring disorders like DM and AD concurrently. These enthused us to investigate the potency of *M. pubescence* against Acetylcholinesterase (AChE), Butyrylcholinesterase (BuChE),  $\alpha$ -Glucosidase and  $\beta$ -Glucosidase enzymes, important pathological targets of AD and DM. **Objectives:** In this study, methanolic extract (MPM) and its derived fractions of *Morinda pubescence* were assessed for their capacities to inhibit target enzymes AChE, BuChE and  $\alpha$ - and  $\beta$ - glucosidases and the active fraction was also screened for its phytoconstituents. **Materials and Methods:** Methanolic extract (MPM) and chloroform fraction (MPC) were evaluated for their inhibitory capacities against AChE and BuChE, and  $\alpha$ - and  $\beta$ -glucosidases besides kinetic analysis of inhibition using methods of Elmann and Shibano, respectively. Antioxidant ability was estimated by DPPH and ABTS radical scavenging activities. Cytotoxicity and neuroprotective abilities were tested by the MTT assay using human neuroblastoma cell lines. Phytochemical screening was done with the aid of spectrophotometric methods. **Results:** The MPC of titled plant had prominent inhibitory potencies against AChE, BuChE,  $\alpha$ - and  $\beta$ -glucosidase enzymes. Kinetic study inferred the pattern of inhibition as mixed. Significant DPPH and ABTS radical scavenging activities were found for MPC (86.95 $\pm$ 1.9 mg TE/g and 3.6 $\pm$ 0.1 mg AAE/g). In MTT assay, the active MPC fraction disclosed remarkable cell viability and neuroprotective abilities in SK-N-SH cells. Phytochemical profiling showed that the fraction MPC had highest amount of flavonoids (41.9 $\pm$ 1.8 mg RE/g dry matter) and phenolics (85.5 $\pm$ 0.98 mg GAE/g dry matter) and had the significant impact on enzyme inhibitory and antioxidant activities. **Conclusion:** The results provided valuable evidence for the potential of chloroform fractions of methanolic extract of *M. pubescence* as prospective material for further development of multifunctional agents to control both Diabetes Mellitus (DM) and Alzheimer's Disease (AD) simultaneously.

**Keywords:** *Morinda pubescence*, Phytochemical, Antioxidant, Anticholinesterase, Antidiabetic, Neuroprotective.

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## INTRODUCTION

Diabetes Mellitus (DM) which affects all genders, in all ages worldwide is a complex metabolic disorder. Over the last few decades, the increasing worldwide prevalence of DM is also of global public health concern as it accelerates ageing in organ systems which in turn lead to premature morbidity and mortality.<sup>[1]</sup>

At present, 387 million individuals live with DM around the world, and it is projected to achieve 592 million by 2035.<sup>[2]</sup> DM is characterized by Postprandial Hyperglycemia (PPHG) and abnormalities in metabolism of carbohydrate, fat and protein owing to insulin resistance or insulin deficiency.<sup>[3]</sup> Elevated PPHG has been linked with onset of diabetic impediments like nephropathy, neuropathy, retinopathy and coronary heart diseases. Decreasing the absorption of glucose through inhibition of  $\alpha$ - and  $\beta$ -glucosidases plays a prominent role in managing PPHG in diabetic patients. Moreover, glucosidase inhibitors reported to be effective against HIV, cancer, hepatitis and heart disorders.<sup>[3]</sup>



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Extensive research efforts have proved DM as a foremost threat for dementia by almost 2-fold or one in ten cases.<sup>[4]</sup> Alzheimer's Disease (AD) contributes 60 to 80% of all dementia. Further, it is the most prevalent age-related progressive neurodegenerative brain disorder with symptoms of memory loss, language deterioration, poor judgment, and functional impairment, loss of independence, emotional distress, and behavioral impairment. The key histopathological hallmarks seen in AD brains are deposits of amyloid-beta (A $\beta$ ), Neurofibrillary Tangles (NFTs), dysfunction of cholinergic neurons and oxidative stress.<sup>[5-8]</sup> Dysfunction of basal forebrain cholinergic neurons results in remarkable depletion and lead to cognitive decline in AD. Currently, the main and putative therapeutic method to alleviate AD symptoms is increasing Acetylcholine (ACh) concentration in the synaptic cleft by inhibition of Acetylcholinesterase (AChE) which hydrolyze ACh in the healthy brain. Recent discoveries indicated that the level of AChE is very low in AD advance cases, while in such instance, Butyrylcholinesterase (BuChE) takes over the hydrolysis and helps to continue AD progression. Therefore, it is of essential importance to search for dual inhibitors of AChE and BuChE as viable therapeutic strategy to treat cognitive dysfunction in AD.<sup>[9,10]</sup> Moreover, numerous studious side effects were noted with FDA approved synthetic drugs due to their peripheral inhibitory activities.<sup>[11-15]</sup> At this juncture, a great attention has been intended to explore natural AChE and BuChE dual inhibitors with an improved safety profile.

Over production of free radicals and progressive decline of the cellular antioxidant defense with aging, impose oxidative stress. The excessive oxidative stress causes neuronal degeneration through damaging cellular lipids, proteins, or DNA. Hence, oxidative stress promotes the pathological hallmarks and progression of degenerative disorders: AD and DM.<sup>[16,17]</sup> Consequently, averting the formation or scavenging the free radicals could be considered as key strategy for AD and DM dual therapy.<sup>[17,18]</sup> Against a background of growing concerns about the toxicity and side effects from currently available therapeutic options for AD and DM, the WHO has also endorsed to explore potent and safer herbal medicines. The growing relevance of herbal medicine as an alternative form of health care is often based on their traditional use in Ayurvedic, Chinese and African medicinal practices. However, there is a shortage in scientific basis for the folkloric applications of medicinal plants. Therefore, pharmacological screening of medicinal plants has become a renewed interest globally. Consequently, during the preliminary screening of plant extracts, *Morinda pubescence* (Rubiaceae) was found to be most potent inhibitor of enzymes involved in DM and AD. Traditional as well as pharmacological applications of *M. pubescence* were noted in Table 1.

According to literature, neither enzyme inhibition nor antioxidant potentials and phytochemical screening of this plant have earlier

been reported elsewhere. In order to fill this gap, current study focused on the evaluation of AChE, BuChE,  $\alpha$ - and  $\beta$ -glucosidase inhibitory, DPPH, ABTS radical scavenging, neuroprotective activities, kinetic analysis of inhibition as well as phytochemical profiling of crude extract and subsequent fractions of titled plant as original contribution in the field of AD and DM dual therapy.

## MATERIALS AND METHODS

### Chemicals and Materials

Human neuroblastoma cells (SK-N-SH) were obtained from National Centre for Cell Sciences (Pune, India). EeAChE from Electric eel (EC 3.1.1.7), EqBuChE from Horse serum (EC 3.1.1.8),  $\alpha$ -glucosidase from *Saccharomyces cerevisiae* (EC 3.2.1.20), and  $\beta$ -glucosidase from almonds (EC 3.2.1.21), ABTS, DPPH, DTNB, pNPG- $\alpha$ , pNPG- $\beta$ , ATCI, BTCl, MTT, Folin-Ciocalteu reagent, galantamine, acarbose, D-Glucono- $\delta$ -lactone and trolox were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents used in this study were of analytical grade or better and were purchased from Merck.

### Plant Material

The titled plant materials (leaves of *M. pubescence*) were collected from Seshachalam hills in Andhra Pradesh, India in September 2013. The voucher specimens vide YVU 61 AGD were identified by Dr. A. Madhusudana Reddy and deposited in the herbarium of Yogi Vemana University. The collected leaves were air dried in the shade for two weeks at RT, and ground into fine powder. The smooth powder was stored in an air-tight container and kept in darkness at -20°C until further use.

### Extraction and Fractionation

For extract preparation, powdered material (150 g) was exhaustively extracted with 90% methanol at 35°C for 48 hr. After filtration, the extracts were concentrated using rotary vacuum evaporator (Heidolph, Germany). The methanolic extracts were suspended in water (50 mL) and sequentially partitioned with chloroform and n-butanol. The residual water solubles were concentrated using rotary vacuum evaporator to get water fraction. Percentage yields of extracts and fractions were calculated (Table 2).

### Cholinesterase Inhibition Assay

The methanolic extract of *M. pubescence* (MPM) and its derived Chloroform (MPC), n-Butanol (MPB) and Water (MPW) fractions were assayed against AChE/BuChE inhibitory activities by Ellman's method.<sup>[24]</sup>

### Glucosidase inhibition Assay

The inhibitory properties of extract MPM and fractions: MPC, MPB, and MPW against  $\alpha$ - and  $\beta$ -glucosidases were measured using protocol reported by Shibano *et al.*<sup>[25]</sup>

## Kinetic Study on Enzyme Inhibition

Kinetic studies for inhibition of enzymes were carried out by measuring enzyme activity at different concentrations of relevant substrate, various concentrations of inhibitor (MPC) and fixed concentration of enzyme using concerned method as described above. Lineweaver Burk plots of  $1/V$  versus  $1/[S]$  and secondary plots of slopes/intercepts Vs inhibitor concentration were constructed with Graph pad prism software.

## ABTS Free Radical Scavenging Assay

Free radical scavenging potential of active fraction MPC on ABTS was determined using procedure developed by Re *et al.*<sup>[26]</sup> Trolox was used as standard, and the results were presented as trolox equivalents.

## DPPH Radical Scavenging Assay

Radical scavenging abilities of test fraction MPC on DPPH were measured as previously reported by Sarikurkcu *et al.*, using ascorbic acid as reference.<sup>[27]</sup>

## Cell Culture experiments

Human neuroblastoma cells (SK-N-SH) were cultured in MEM to reach approximately 80% confluence as reported earlier<sup>[28]</sup> and used for *in vitro* experiments.

## MTT Assay

To predict cytotoxicity of active fraction MPC, MTT assay was performed using SK-N-SH cells as disclosed in our recent studies.<sup>[29]</sup>

## Neuroprotectivity assay in SK-N-SH cells

Neuroprotective potency of fraction MPC against  $H_2O_2$  induced oxidative injury in SK-N-SH cells was determined using MTT colorimetry method.<sup>[29]</sup>

## Phytochemical Analysis

### Total Phenolic Contents (TPC)

TPC was determined using Folin-Ciocalteu (FC) reagent and expressed as mg of gallic acid equivalents (mg GAE/g).<sup>[30]</sup>

### Total Flavonoid Contents (TFC)

TFC was quantified and expressed as mg of rutin equivalent (mg RE/g) using aluminum chloride colorimetric method.<sup>[31,32]</sup>

### Total Tannins Contents (TTC)

TTC was estimated as mg of Catechin equivalents (mg CE/mL) using Vanillin HCl method.<sup>[33]</sup>

## Total Terpenoids Contents (TTrC)

Quantities of TTrC was noted as linalool equivalents (mg LE/g extract) by an assay of Narayan Ghorai *et al.*<sup>[34]</sup>

## Total Alkaloid Content (TAC)

Quantitative analysis of TAC was carried out using BCG solution<sup>[35]</sup> and recorded as atropine equivalents (mg AE/g extract).

## RESULTS

### Cholinesterase inhibitory activity

In recent years, plants have proven to be an important source for the inhibitors of cholinesterases (AChE and BuChE) that are useful in symptomatic relief in AD. Presently, the methanolic extract of *M. pubescence* (MPM) and its derived chloroform, n-butanol and water fractions (MPC, MPB, and MPW) were tested towards AChE and BuChE inhibition potency with the modified Ellman's method in a 96-well microplate reader using Galantamine as reference (Ellman *et al.* 1961). All the screened samples exhibited dose-dependent inhibitory abilities on AChE and BuChE activities at 15, 30, 90 and 150  $\mu\text{g/mL}$  concentrations (Figure 1). The results in the form of  $IC_{50}$  values were summarized in Table 2. The methanolic extract MPM showed strong inhibition abilities against AChE and BuChE with  $IC_{50}$  values in micrograms range that to below 65  $\mu\text{g/mL}$ . Among the fractions tested the chloroform fraction MPC exhibited most potent activity with  $IC_{50}$  values  $32.70 \pm 5.6$  and  $63.14 \pm 20.8$   $\mu\text{g/mL}$  for AChE and BuChE, respectively. Regarding n-butanol and water fractions, moderate to low activity against both the enzymes was recorded.

### Inhibition of $\alpha$ - and $\beta$ -glucosidase enzymes

In this study, inhibitory abilities of the methanolic extract and subsequent fractions of titled plant toward  $\alpha$ - and  $\beta$ -glucosidases were evaluated as depicted by Shibano *et al.* with minor alterations<sup>[25]</sup> using acarbose and D-Glucono- $\delta$ -lactone as references. In the *in vitro* experiment, the used substrate pNPG- $\alpha$  or pNPG- $\beta$  was hydrolyzed by glucosidases and release a color agent p-nitrophenol that can be monitored at 415 nm. The calculated  $IC_{50}$  values against  $\alpha$ - and  $\beta$ -glucosidases were shown in Table 2 (Figure 1).

The *in vitro* inhibitory studies demonstrated that both methanolic extract MPM have strong  $\alpha$ - and  $\beta$ -glucosidase inhibitory activities with  $IC_{50}$  values less than 25  $\mu\text{g/mL}$ . Among all the fractions screened, highly active MPC had with  $IC_{50}$  values of  $10.29 \pm 0.8$  and  $55.12 \pm 2.09$  against  $\alpha$ -glucosidase and  $\beta$ -glucosidase, respectively. It is also interesting that the MPM and MPC provided stronger inhibitory activities on  $\alpha$ -glucosidase than reference compound acarbose. According to data, n-butanol fraction MPB was considered to be moderate inhibitor of  $\alpha$ - and  $\beta$ -glucosidases, on the other hand, water fraction MPW was less active.

## Kinetics of inhibition

The most active fraction MPC was chosen for further kinetic analysis of inhibition towards all target enzymes. First, the initial velocity of each enzyme was monitored at 15, 30 and 90 µg concentrations of fraction MPC using different concentrations (0.1-0.5 mM) of the corresponding substrate. The Lineweaver-Burk plots (Figure 2), double reciprocal plots of initial velocity (1/v) and substrates concentration (1/s) revealed the pattern of increasing slopes and intercepts with increase of inhibitor concentration and the falling intersect points in the upper left quadrant indicating a mixed-type inhibitory behavior of active fractions. This implies that inhibitor binds to both free Enzyme (E) and Enzyme-Substrate complex (E+S) and accordingly, secondary plots gave  $K_{i1}$  and  $K_{i2}$  inhibition constants as shown in Table 3.

## Antioxidant activity

The most active fraction MPC was assessed for their ABTS and DPPH radical scavenging abilities of using trolox and ascorbic

acid, respectively as reference and represented as mg Trolox and Ascorbic acid Equivalents.

The tested fraction MPC exhibited strong ABTS radical scavenging capacity with 86.95±1.9 mg Trolox Equivalents per gram. Regarding DPPH radical scavenging activity, MPC disclosed modest scavenging abilities as 3.6±0.1 mg Ascorbic acid Equivalents per gram, respectively as shown in Table 4.

## Cell viability

The cytotoxic effect of fraction MPC was measured in SK-N-SH cell line by MTT assay. After treating the SK-N-SH cells with fraction MPC at 50, 100, 200 and 400 µg for 24 hr, the cell viability was measured (Figure 3) represents cell viability (%) after treatment of the fraction MPC comparing with control cells. MPC showed concentration dependent cell viability.

## Neuroprotective activity

The neuroprotective capacities of MPC against H<sub>2</sub>O<sub>2</sub> induced cell death in SK-N-SH cells were investigated by MTT assay.

**Table 1: Traditional uses and pharmacological activities of *M. pubescence*.**

Plant name	Regional name	Traditional uses	Activities
<i>Morinda pubescence</i>	Togaru and Maddi.	Astringent, deobstruent, emmenagogue and relieve pain in the gout. <sup>[19,20]</sup>	Anticancer, antimicrobial, cytotoxic, anti-plasmodial, analgesic, hypotensive, hepatoprotective, anti-inflammatory and anticonvulsant effects. <sup>[21-23]</sup>

**Table 2: % yield of plant leaves and AChE, BuChE, α- and β-glucosidases inhibitory activities (IC<sub>50</sub> Values) of extract (MPM) and fractions (MPC, MPB, MPW).**

Plant	Extract/ fractions	% of yield	IC <sub>50</sub> Values (µg/mL)			
			AChE	BuChE	α-glucosidase	β-glucosidase
<i>M. pubescence</i>	Methanol	14.59	56.17±4.7	51.64±5.82	13.99±1.02	24.65±0.2
	Chloroform	3.5	32.70±5.6	63.14±20.8	10.29±0.8	55±22.09
	n-Butanol	6.1	218.37±21.2	208.74±125	149.12±4.22	155.57±6.3
	Water	8.49	179.41±35.3	159.37±7.5	424.02±75.7	227.86±53.9
Galantamine	-		0.77±0.09	8.1±0.02	-	-
Acarbose	-		-	-	117.20±0.017	-
D-Glucono-δ-lactone	-		-	-	-	10.68±0.0058

**Table 3: Kinetic study of MPC on AChE/BuChE/α- glucosidase and β- glucosidase inhibition.**

Plant	Fraction	Enzymes	Inhibition Type	Inhibition constant (ki(µg/mL))	
				Ki <sub>1</sub>	Ki <sub>2</sub>
<i>M. pubescence</i>	Chloroform	AChE	Mixed	37.41	208.4
		BuChE	Mixed	38.1	93.02
		α- glucosidase	Mixed	13.59	58.69
		β- glucosidase	Mixed	46.76	236.5



After exposure to 1.0 mM H<sub>2</sub>O<sub>2</sub> for 8 hr, the cell survival rate of SK-N-SH cells was markedly reduced to 44.65%. The sensitivity towards H<sub>2</sub>O<sub>2</sub> was noticed in concentration and time-dependent manner. The protective efficiency of fraction MPC at different concentrations of 50, 100, 200 and 400 µg against H<sub>2</sub>O<sub>2</sub> induced oxidative stress was expressed as corresponding cell viability in Figure 4. Tested fraction MPC excelled remarkable protection capabilities with cell viabilities ranged from 57.96-165.23% in dose response way. Very interestingly, MPC showed strong neuroprotective effect at all concentrations.

### Phytochemical analysis

Natural phytochemicals usually protect plants from disease, pathogenic attack and damage from environmental hazards. Bioactivities exhibited by plants usually owing to the interactions of their constituents with specific metabolic pathways. Therefore, phytochemical profiling of active chloroform fraction MPC of titled plant was made using standard procedures. Screening results indicated the occurrence of phenolics, flavonoids, tannins and alkaloids in MPC. However, MPC found to be rich in Flavonoid Contents (TFC) and Phenolic Contents (TPC). Total phenolic contents were quantified as milligram gallic acid

equivalents per gram (GAE/g extract). The TFC estimated was expressed as milligram rutin equivalents per gram (RE/g extract). The highest amounts of flavonoids (41.9±1.8 mg RE/g dry matter) and phenolics (85.5±0.98 mg GAE/g dry matter) were found in MPC. However, moderate quantities of tannins (21.59±1.68 mg CE/g) and alkaloids (9.55±1 mg AE/gm) were noticed in MPC while lowest in terpenoid (0.889±0.08 mg LE/gm) content were shown in Table 5.

### DISCUSSION

AD and DM are common disorders in the aged people. Though they renowned as two individual diseases, in recent times DM has known to contribute majorly to the development of AD as insulin resistance is associated with cognitive decline and memory deficits, and hence, AD was referred as type 3 diabetes. Supportingly, recent studies has also revealed that insulin plays a crucial role in the key pathological issues of AD like phosphorylation of tau and formation of amyloid plaques.<sup>[36]</sup> Currently, the methanolic extract of *M. pubescence* was fractionated using different solvents to acquire various phytoconstituent rich fractions, evaluated for their potential towards dual therapy of AD and DM through

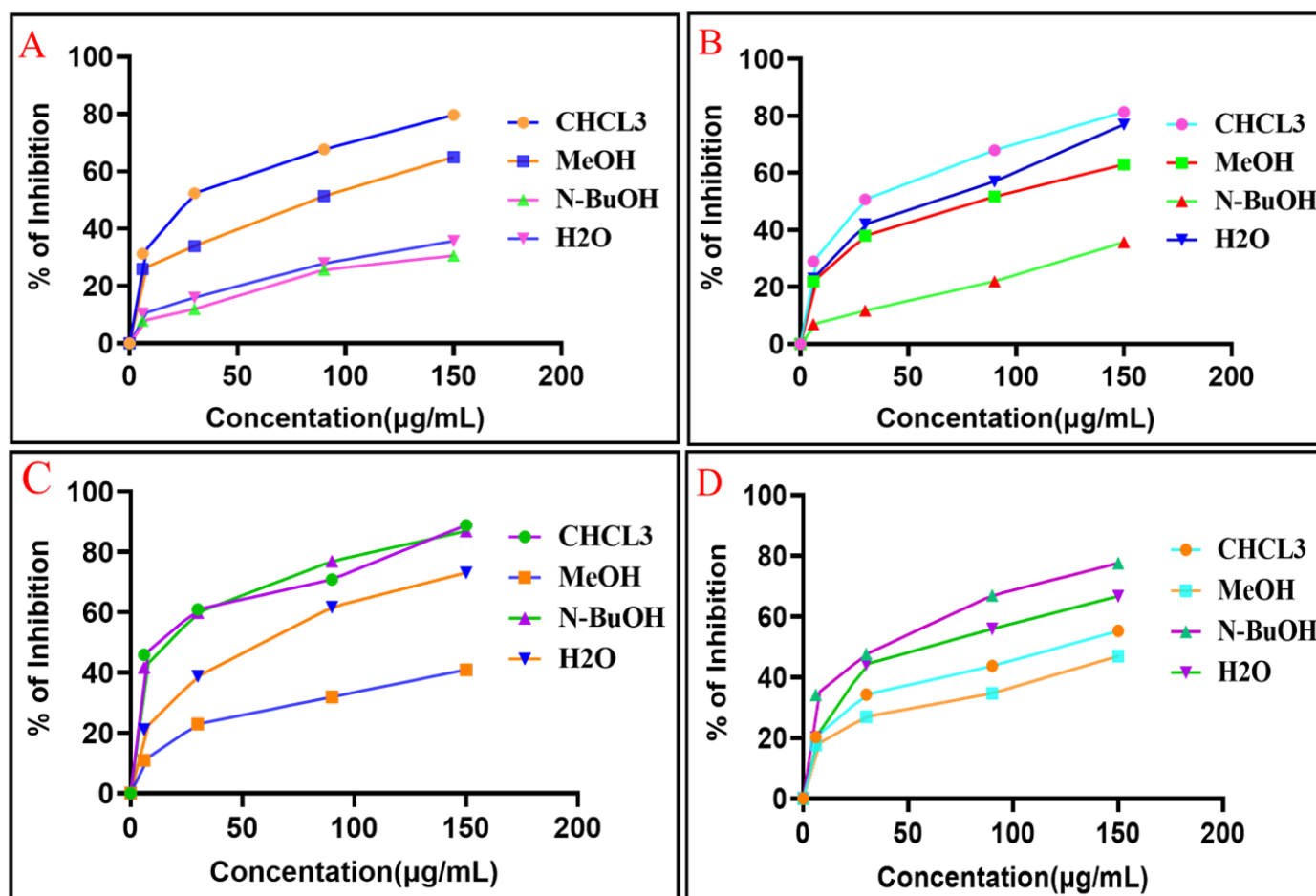
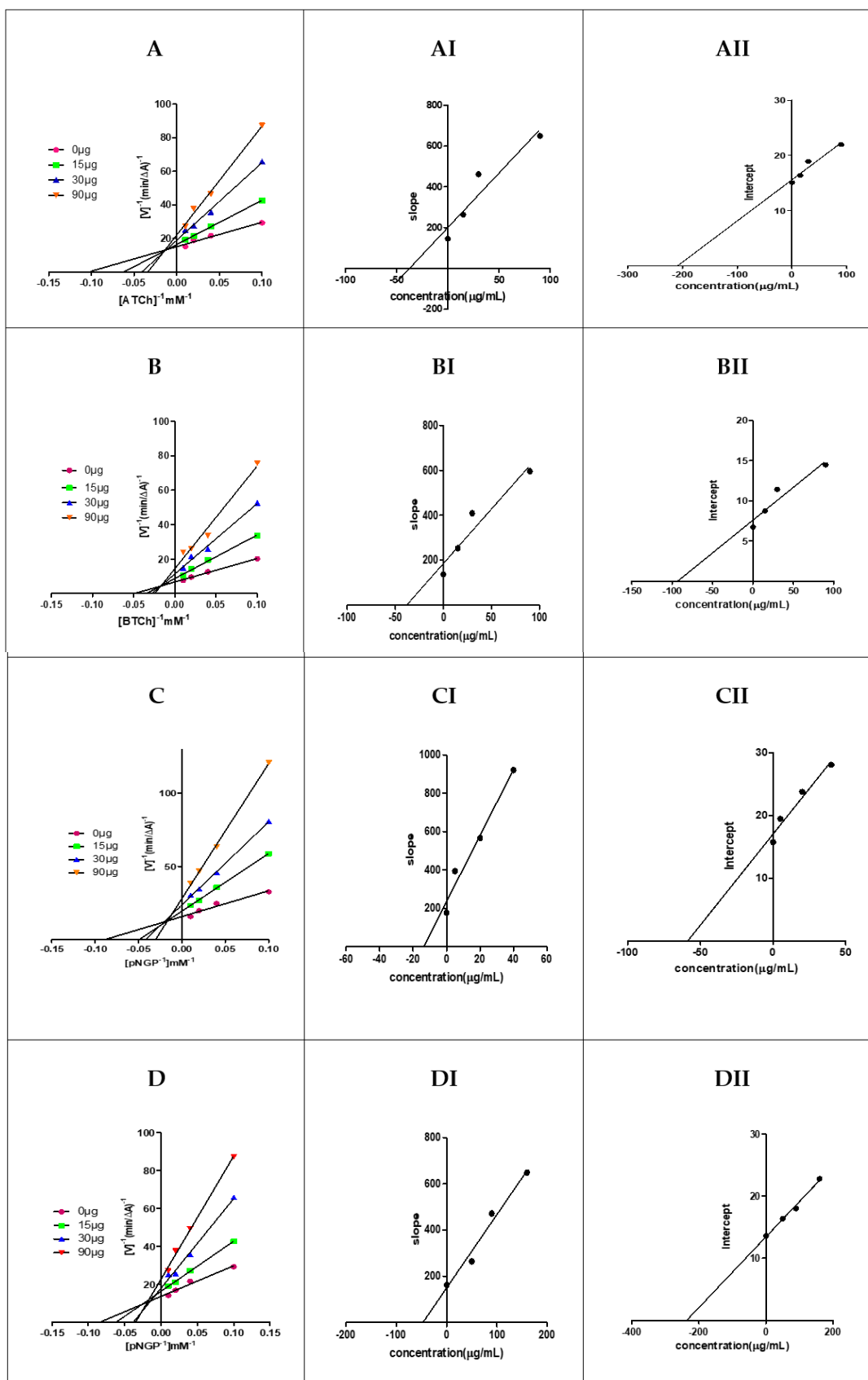
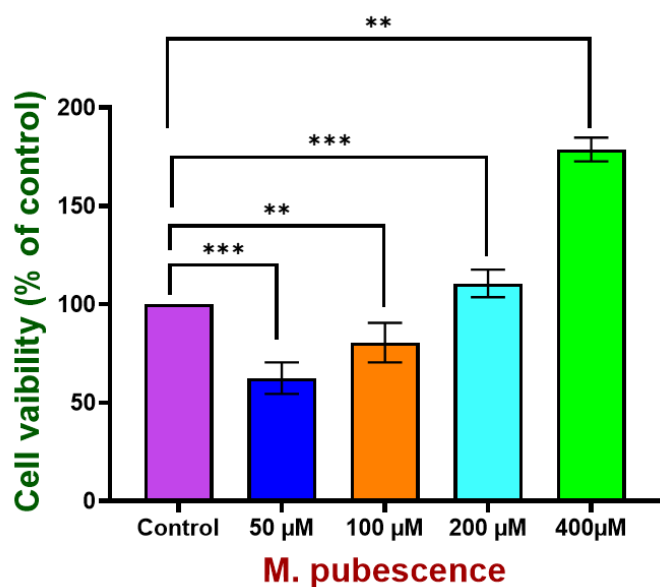


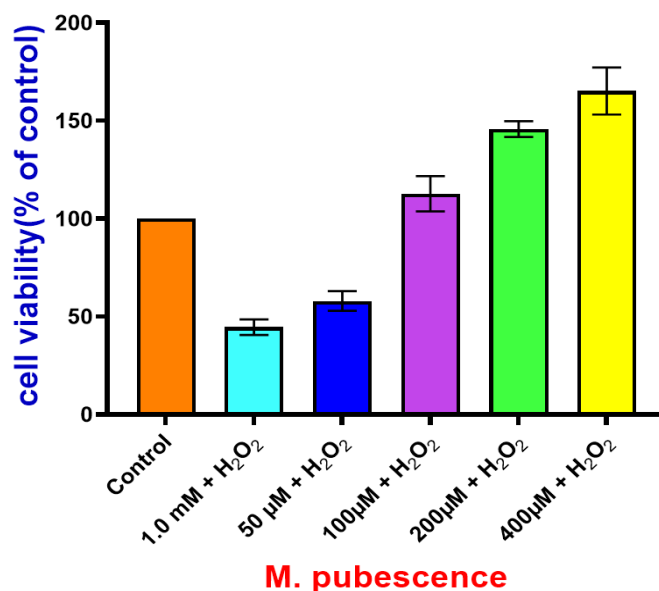
Figure 1: The percentage of inhibition exerted by *M. pubescence* on the activity of cholinesterases (AChE and BuChE) and α- and β-glucosidases at 15, 30, 90 and 150 µg/mL concentrations.



**Figure 2:** Steady-state inhibition of AChE (A), BChE (B),  $\alpha$ -Glucosidase (C) and  $\beta$ -Glucosidase (D) by most active fraction (GAC) from *M. pubescence* (Left) Lineweaver Burk plot of reciprocal of initial velocities versus reciprocal of acetylthiocholine iodide concentrations (0.1-0.5 mM) in the absence and presence of BAC at 15  $\mu$ g, 30  $\mu$ g, 90  $\mu$ g and  $\mu$ g; (right) secondary plots of the Lineweaver Burk plot, slope versus various concentrations of MPC (I) regarding inhibition constant  $K_i$  and intercept versus various concentrations of MPC (II) regarding inhibition constant  $K_{i2}$ .



**Figure 3:** Neurotoxic effects of chloroform fraction of methanolic extract of *M. pubescence* on SK-N-SH cells (human neuroblastoma cell line). Bar chart show the percentage of cell viability in the presence or absence (control) of indicated concentrations of *M. pubescence*. Cell viability corresponding to control cells was considered as 100%. \* $p < 0.05$  versus 100% cell viability (one-way ANOVA test).



**Figure 4:** Neuroprotective activity of chloroform fraction from methanolic extract of *M. pubescence* on SK-N-SH cells (human neuroblastoma cell line) against H<sub>2</sub>O<sub>2</sub> induced cell death in SK-N-SH cells in MTT assay. \* $p < 0.05$  versus H<sub>2</sub>O<sub>2</sub> treatment.

their inhibitory activities against AChE, BuChE and  $\alpha$ - and  $\beta$ -glucosidase enzymes for the first time.

The usual 4:1 ratio of AChE and BuChE in healthy human brain was reported to alter in AD patients since activity of AChE decline up to 45% and increment of BuChE activity up to 2-fold during the AD progression. In this context, BuChE takes a major role in regulating the ACh level. Consequently, dual inhibitors that inhibit both AChE and BuChE simultaneously have tremendous benefits in the AD treatment.<sup>[37]</sup> The chloroform fraction MPC showed significant inhibitory potentials against both AChE and BuChE and thus regarded as AChE and BuChE dual inhibitor. Literature stated that plant extracts with dual AChE and BuChE inhibition potentials may be of suitable to cure the patients with moderate AD.<sup>[38]</sup> Moreover, it was also reported that dual inhibitors were capable of reducing cleavage of amyloid precursor protein and aggregation of A $\beta$  which were key factors in AD pathology.<sup>[39]</sup> Hence, as dual inhibitors of AChE and BuChE, the active fraction MPC may be of more useful in treating AD.

Decreasing post prandial hyperglycemia through inhibition of  $\alpha$ - and  $\beta$ -glucosidases ( $\alpha$ - and  $\beta$ -Glu) carbohydrate hydrolyzing enzymes was acknowledged as main therapeutic approach for treating DM. Interestingly, MPM and MPC provided stronger inhibitory activities on  $\alpha$ -glucosidase than reference compound acarbose. MPM and MPC were also found to inhibit  $\beta$ -glucosidase activity significantly. Therefore, MPM and MPC were regarded as dual inhibitors of  $\alpha$ - and  $\beta$ -glucosidases, evidently have excellent antidiabetic potency. Dual  $\alpha$ - and  $\beta$ -glucosidase inhibitors were reported to have better clinical potency against DM without

**Table 4: ABTS and DPPH Free Radical scavenging activity of chloroform fraction.**

Plant	ABTS mg TE /g	DPPH mg AAE / g
<i>M. pubescence</i>	86.95±1.9	3.6±0.1

All values mean±standard deviation of triplicates; TE: Trolox Equivalents, AAE: Ascorbic Acid Equivalents.

remarkable side effects. Consequently, the most potent fraction MPC with good balance of  $\alpha$ - and  $\beta$ -glucosidase inhibitory activities may represent better therapeutic option to treat DM.

Over production of ROS reported to inactivate enzymes and damage vital cellular organelles and membranes, consequently, turn into causative factor for several disorders including AD and DM. Also, imbalance in oxidative defense mechanism plays a special role in onset of degenerative disorders like AD and DM.<sup>[40,41]</sup> Therefore, scavenging of ROS has renowned as a worthy strategy in the drug discovery for AD and DM. Recently, natural sources like plants and plant products with better biocompatibility and lower side effects have gained ample recognition as antioxidants.<sup>[42,43]</sup> The stronger ABTS and DPPH scavenging activity of fraction MPC indicating its remarkable capacities to protect from oxidative damage by ROS, which is an additional benefit in treating AD and DM simultaneously.

Interestingly, in MTT assay, at higher concentrations, fraction MPC has excelled remarkable neuroprotection capabilities in human neuroblastoma SK-N-SH cells against H<sub>2</sub>O<sub>2</sub> induced oxidative stress than control. As per earlier reports, it is reasonably assumed that phytoconstituents of tested fraction may probably promote cell survival or delay the natural death of

**Table 5: Quantitative phytochemical analysis of the active chloroform fractions from *M. pubescence*.**

plant	TPC mg GAE / g	TFC mg RE/ g	TTC mg CE/g	TTRC mg LE/g	TAC mg AE/g
<i>M. pubescence</i>	85.5±0.98	41.9±1.8	21.59±1.68	0.889±0.08	9.55±1

TPC: Total Phenolic Content, TFC: Total Flavonoid Content, TTC: Total tannin content, TTRC: Total Terpenoid Content, TAC: Total Alkaloid Content, GAE: Gallic Acid Equivalents, RE: Rutin Equivalents, CE: Catechin Equivalents, LE: Linalool Equivalents, AE: Atropin equivalents.

neuronal cells.<sup>[28]</sup> The increase in cell viability by averting H<sub>2</sub>O<sub>2</sub> induced oxidative cell death indicating that this fraction might be potential oxidative suppressor. In cell viability assay, MPC showed concentration dependent cell viability. The higher cell viability by MPC than the control cells with increasing concentrations indicated that fraction stimulated the growth and proliferation of cells with increasing concentration and nontoxic to SK-N-SH cells.

Phytochemical analysis offers fundamental knowledge about therapeutic significance of a plant extract due to synergistic health promoting effect of secondary metabolites such as phenolics, flavonoids, tannins, terpenoids, alkaloids, carotenoids etc.<sup>[44]</sup> Quantitative analysis of secondary metabolites inferred that the active fraction MPC of *M. pubescence* found to enrich highest TFC and TPC. Flavonoids, a prominent group of secondary metabolites have wide spectrum of pharmacological activities such as anti-cancer, anti-diabetic, antioxidant, antimicrobial, anti-inflammatory, anti-cholesterolemic etc. These metabolites were also proved to be strong free radical scavengers due to their hydrogen or electron donating abilities and chelating nature with transition metals.<sup>[45]</sup> Flavonoids were also proved to be potent in precluding or reducing the progression of AD by inhibiting amyloid- $\beta$  peptide and tau protein aggregation.<sup>[46]</sup> Flavonoids were also found to inhibit target enzymes involved in the treatment of AD and DM. Plant phenolics were also considered as potent antioxidants through multiple mechanisms like scavenging, adsorbing, quenching, decomposing and neutralizing free radicals and thus have therapeutic potential against AD and DM.<sup>[47]</sup> Hence, a strong correlation between enzyme inhibitory and antioxidant activities and TFC and TPC may be possible. Finally, with the aid of literature and the present results, it can be recommended that phenolics and flavonoids alone or in combination are the major contributors for the antioxidant and enzyme inhibitory activities of MPC. Therefore, with aforementioned outcomes, chloroform fraction of methanolic extract of *M. pubescence* could be proposed as attractive and nontoxic agents with consistent multipotent therapeutic potential for the dual therapy of AD and DM.

## CONCLUSION

Taken together, the methanol extract and its chloroform fractions of *M. pubescence* are very strong mixed type inhibitors of AChE, BuChE,  $\alpha$ -Glucosidase and  $\beta$ -Glucosidase enzymes, important pathological targets of AD and DM. Further, chloroform fraction

could be considered as a significant source of natural antioxidants with potent neuroprotective abilities. In phytochemical analysis, chloroform fractions have a rich mixture of phenolics and flavonoids that offer a remarkable enzyme inhibitory, antioxidant and neuroprotective properties. To the best of our knowledge, we herein divulge the first report on cholinesterase and glucosidase inhibitory, radical scavenging and neuroprotective activity of *M. pubescence*. In conclusion, the present study demonstrates the potential of chloroform fraction of methanolic extract of leaves of *M. pubescence* as multifunctional therapeutic remedy for the AD and DM dual therapy. The study warrants further investigations to isolate and characterize the active substances from the active fraction and to explore their potential in combating degenerative diseases such as AD and DM.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## ABBREVIATIONS

**MPC:** *Morinda pubescence*; **AD:** Alzheimer's Disease; **DM:** Diabetes Mellitus; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **ABTS:** 2,2-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid; **AChE:** Acetylcholinesterase; **BuChE:** Butyrylcholinesterase; **PPHG:** Postprandial hyperglycemia; **A $\beta$**  Amyloid-beta; **NFTs:** Neurofibrillary tangles; **DTNB:** 5,5'-dithiobis-(2-nitrobenzoic acid); **MTT:** (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide); **DMSO:** Dimethyl sulfoxide; **ANOVA:** Analysis of variance.



## AUTHORS' CONTRIBUTION

RBZ: Conceptualization, Methodology, Investigation, Data curation, Writing, Original draft preparation and Visualization; MP: Investigation and Methodology; JBS: Methodology, Writing and Editing; YRK: Investigation and draft preparation; SLM: Investigation; KPV: investigation; SV: Resources and Methodology; RV: Resources and Methodology; DAG: Project administration, Supervision, Conceptualization, Methodology, Resources, Writing, Reviewing, Editing and Funding acquisition.

## SUMMARY

Extensive epidemiological and clinical studies revealed that Alzheimer's Disease (AD) and Diabetes Mellitus (DM) are most likely to appear simultaneously in aged people as DM is a major risk factor for AD. As a result, development of multi-targeted agents found to be one of the best options to combat co-occurring disorders like DM and AD simultaneously. Therefore, the present study aimed to assess the multifunctional potencies of *Morinda pubescence* towards AD and DM dual therapy. The results revealed that the chloroform fraction (MPC) of methanolic extract of titled plant had prominent inhibitory potencies against AChE, BuChE,  $\alpha$ - and  $\beta$ -glucosidase enzymes. Kinetic study inferred the pattern of inhibition as mixed. Significant DPPH and ABTS radical scavenging activities were found for MPC ( $86.95 \pm 1.9$  mg TE/g and  $3.6 \pm 0.1$  mg AAE/g). In MTT assay, the active MPC fraction disclosed remarkable cell viability and neuroprotective abilities in SK-N-SH cells. Phytochemical profiling showed that the fraction MPC had highest amount of flavonoids ( $41.9 \pm 1.8$  mg RE/g dry matter) and phenolics ( $85.5 \pm 0.98$  mg GAE/g dry matter) and had the significant impact on enzyme inhibitory and antioxidant activities. These results indicated that the chloroform fraction of methanolic extracts of *M. pubescence* has substantial antioxidant as well as inhibitory capacities on target enzymes AChE, BuChE,  $\alpha$ - and  $\beta$ -glucosidase which suggest their multifunctional effectiveness for AD and DM dual therapy and encourage further investigations to isolate and characterize the active substances from the active fraction and to explore their potential in combating degenerative diseases such as AD and DM.

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