

In vitro & ex vivo Acetylcholinesterase Inhibitory Activity of *Morinda citrifolia* Linn (Noni) Fruit Extract

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ABSTRACT

Background: Psychological problem is a key medical issue for numerous neuropsychiatric and neurodegenerative diseases, for example, schizophrenia, Alzheimer's, dementia, seizure and Parkinsonism. *Morinda citrifolia* (Noni) has been utilized for a considerable length of time to cure or counteract assortment of diseases by conventional therapeutic professionals in Hawaii and Polynesia. **Objective:** The present study is focused to identify the neuroprotective activity of *Morinda citrifolia* fruit extract (MCFE) on *in vitro* and *ex vivo* animal model by inhibition of acetylcholinesterase (AChE), an enzyme target used for the treatment of Alzheimer's disease. **Methods:** Acetylcholinesterase inhibition assay was performed by *in vitro* & *ex vivo* methods as described by Ellman *et al.* *In vitro* antioxidant assay of the extract was performed by DPPH free radical scavenging activity & nitric oxide scavenging activity. **Statistical analysis used:** Statistical analysis was carried out using non linear regression analysis for plotting the line of best fit for the observed values using Graph-Pad Prism software. **Results:** By performing *in vitro* antioxidant assay the IC₅₀ value of the standard quercetin was found to be 46.22 µg/ml as compared to the MCFE which has an IC₅₀ value of 43.14 µg/ml for DPPH free radical scavenging activity. Similarly the IC₅₀ value of the standard ascorbic acid was found to be 81.85 µg/ml as compared to the MCFE which has an IC₅₀ value of 148.0 µg/ml for nitric oxide scavenging activity. Acetylcholinesterase inhibition assay was performed by *in vitro* method and the IC₅₀ value of MCFE and neostigmine was found to be 31.84 µg/ml & 19.71 µg/ml respectively. **Conclusions:** The present study investigated the neuroprotective activity of MCFE and it was identified by both *in vitro* and *ex vivo* techniques that the phytoconstituents has the ability to improve the learning and memory function by inhibiting the acetylcholinesterase.

Key words: *Morinda citrifolia*, Noni, Acetylcholinesterase, Neuroprotective activity, *in vitro*, *ex vivo*.

Key Messages: The present study investigated the neuroprotective activity of *Morinda citrifolia* and it was identified by both *in vitro* and *ex vivo* techniques that the phytoconstituents in the plant can improve the learning and memory function by inhibiting the acetylcholinesterase. Further the antioxidant potential of the plant was also evident from the DPPH and nitric oxide scavenging activity. Future studies may be designed for chronic administration of *Morinda citrifolia* to further investigate the effect on *in vivo* experimentation and also to identify the safety and efficacy parameters at both preclinical and clinical stages.

INTRODUCTION

Psychological problem is a key medical issue for numerous neuropsychiatric and neurodegenerative disorders like schizophrenia, Alzheimer's, dementia, seizure and Parkinsonism. Cholinergic framework is considered as one of the key part in the control of learning and memory functions. Cholinesterase inhibitors increase the cholinergic transmission specifically by preventing acetylcholinesterase (AChE) which hydrolyses acetylcholine.¹ Incredible significance has been given for the identification of neurotransmitter component concerned in the characteristic indication of neurological and psychiatric disorders.² Acetylcholinesterase has been ended up

being the most conceivable remedial focus for the indicative management of Alzheimer's disease. Acetylcholinesterase is a constituent of the α/β hydrolase protein super family, playing a significant role in acetylcholine-interceded neurotransmission. Lately, the part of AChE inhibitors was reassessed from neurotoxins to neuron-defensive in the disorders with disruption in cholinergic transmission.³

Besides, research in animal models has demonstrated that the combination therapy with memantine and the AChE inhibitors can enhance memory than either treatment alone with the single drug. In the clinical setting, blend treatment

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with memantine and an AChE inhibitor has proved to be an appreciated progress for the treatment of patients with Alzheimer's disease.⁴ Mankind is gifted with plenty of plant based natural sources of drugs. The various collections of bioactive nutrients available in these natural food plays an essential function in avoidance and healing of various neurodegenerative diseases, such as Alzheimer's, Parkinsonism and other neuronal disorders. Aggregated confirmation recommends that the phytoconstituents, for example, polyphenolic cell reinforcements found in natural products, vegetables, herbs and nuts, may conceivably frustrate neurodegeneration, and improve memory and intellectual capacity.⁵

Phytochemicals from medicinal plants assume a fundamental part in maintaining the mental balance regulated by the function of receptors for the major inhibitory neurotransmitters. As per study, the natural pharmaceuticals utilized as a part of Ayurvedic and Chinese medicines contain various phytoconstituents with neuroprotective impact which may be helpful in neuropsychiatric and neurodegenerative disorders.⁶

Morinda citrifolia Linn belongs to the family Rubiaceae and habituated to Sub-himalayan tracts, Darjeeling, Konkan and Andamans. The Indian Mulberry is commonly known as ashvika, akshi & atchy in Ayurveda & nuna, togaru in Siddha.⁷ *Morinda citrifolia* (Noni) has been utilized for a considerable length of time to cure or counteract assortment of diseases by conventional therapeutic professionals in Hawaii and Polynesia. A complete review on *Morinda citrifolia* was performed by Assi *et al* in 2016 which described the antimicrobial and antiseptic activity, antifungal activity, antioxidant activity, anti-inflammatory activity, anti-arthritis activity, anti-cancer activity, antidiabetic activity, wound healing activity, memory enhancing activity, anxiolytic and sedative activity, analgesic activity, gastric ulcer healing activity, antiemetic activity, gout and hyperuricemia healing activity, immunity enhancing activity, anti-viral activity, anti-parasitic activity, anti-tuberculosis activity, osteoporotic and otoscopic enhancer. These activities were carried out to the extent of *in-vitro*, *in-vivo* & clinical trial stages.⁸

The neuroprotective effect of an ethyl acetate fruit extract of *Morinda citrifolia* (Rubiaceae) Linn at doses of 200 and 400 mg/kg, po was contemplated by Muralidharan *et al.*⁹ Past study by Pachauri *et al.* was intended to examine impact of Noni on memory, cerebral blood flow (CBF), oxidative stress and AChE action in scopolamine incited amnesia mice model.¹⁰ Harada *et al.* inspected the impact of the juice of *Morinda citrifolia* (Noni juice) on the brain damage brought about by ischemic stress in mice.¹¹ In our past study we reported the antiepileptic activity of *Morinda citrifolia* Linn fruit extract against seizures instigated by Maximal Electro Shock (MES) technique in rats.¹²

Köktürk *et al* assessed the neuroprotective impacts of *Morinda citrifolia* L. (Rubiaceae), regularly known as Noni, and memantine on hydrocephalus-actuated neurodegenerative disorders.¹³ Pandey *et al* showed the antidopaminergic impact of *Morinda citrifolia* Linn.¹⁴ The discoveries by Muto *et al* recommended that the association of Noni fruit juice shields brain from stress-instigated impedance of psychological capacity and that this defensive impact might be identified with change in stress-initiated diminishes in vein thickness in the hippocampal dentate gyrus.¹⁵ Harada *et al* recommended that Noni juice could encourage insulin discharge after ischemic stress and may reduce the advancement of glucose intolerance. These components may add to the neuronal defensive impact of Noni juice against ischemic stress.¹⁶ Pachauri *et al* proposed the useful impacts of Noni organic product against streptozotocin-induced memory weakness, which might be ascribed to enhanced mind vitality system, cholinergic neurotransmission and antioxidative activity.¹⁷ One of the previous study reported *in silico* acetylcholinesterase inhibitory activity of *Morinda citrifolia*.¹⁸ The present study is focused to identify the neuroprotective activity of MCFE on *in vitro* and *ex vivo* animal

models by inhibition of acetylcholinesterase, an enzyme target used for the management of Alzheimer's disease.

SUBJECTS AND METHODS

Chemicals

1,1 Diphenyl -2-picryl hydrazyl hydrate (DPPH), was obtained from Himedia, Mumbai, quercetin, ascorbic acid, was obtained from SRL, Mumbai, trichloroacetic acid, Acetylthiocholine iodate, 5,5 - Dithiobis [2-nitrobenzoic acid], sodium nitroprusside, Griess reagent, Bovine serum albumin, Folin catecheau reagent were purchased from SD Fine Ltd, Mumbai. All other chemicals used in the study was procured from local suppliers and of analytical grade.

Plant Extract

The full spectrum standardized extract of *Morinda citrifolia* fruit was obtained from Amsar Goa Pvt Ltd, Goa. The plant was authenticated by Dr Laxmi Morajkar, Head Ayurveda Division (Voucher specimen Number: AGPL/039/13-14). *Morinda citrifolia* fruits were shade dried and kept in an air tight container. Extraction was carried out with water and ethanol in a ratio of 80:20 by a simple maceration technique. The hydro alcoholic solvent of 1 liter was added to the shade dried plant powder of 100 g and placed on a mechanical shaker for a period of 4 hours. Then the solution was filtered through Whatman No.1 filter paper. The filtrate concentrated using flash evaporator and further processed to dryness in vacuum desiccators. 4.5 g was the percentage yield of the hydroalcoholic extract of *Morinda citrifolia*.

Animals

The *ex-vivo* study was performed using Swiss albino mice of 20-25 g. The Swiss albino inbred colonies of mice was purchased from Venkateshwara enterprises, Bangalore. The mice were adjusted and assimilated to controlled laboratory condition of Center of Toxicology and Developmental Research (CEFT) with temperature (23±2°C), humidity (50±5%) and 12 h light dark cycles. The experimental animals were randomized in to control and treatment groups and were placed in a sanitized polypropylene cages containing sterile paddy husk as bedding. The animals were freely accessed to standard pellets and water ad libitum. All the studies conducted were approved by the Institutional Ethical Committee, Sri Ramachandra University, Chennai, (IAEC/XLIII/SRU/424/2015).

In vitro antioxidant activity

DPPH radical scavenging assay

The free radical scavenging activity of the plant extracts was examined by the tactic given by Yohozowa *et al* using staining of ethanolic solution of 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH).¹⁹ Quercetin was used as a positive control and the ethanolic solution of DPPH was used as a control. The reaction mixture containing 1.9 ml of freshly prepared DPPH (200µM in ethanol) with various concentrations of the sample MCFE (25-800 µg/ml) was shaken. Discolorations were measured at 517 nm by using UV Spectrophotometer after incubation of mixture for 20 min at room temperature. The level of discoloration indicates the free radical scavenging effectiveness of the substances. The Percentage of DPPH free radical scavenging activity was calculated by the following formula:

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

Where A_{blank} , A_{sample} and A_{control} are the absorbance of blank, sample and control, respectively. The half maximal inhibitory concentration (IC_{50}) was calculated from the Prism (GraphPad 5.0, Dose-response - Inhibition) curve obtained by plotting the percentage inhibition against the log concentration.

Nitric oxide radical scavenging assay

The sample MCFE (25-800 mg/ml) with nitric oxide was determined by the nitrite detection method described by Alderton *et al.*²⁰ The nitric oxide was generated from 100 nM sodium nitroprusside in 10 mM phosphate buffer (pH 7.4) and measured by Griess reaction. The reaction mixture containing 10 mM sodium nitroprusside in phosphate buffer. Griess reagent was added to the aliquot to homogenize after the incubation at 37°C for 4 hours. The amount of nitrite formed was measured at 546 nm and referred to the absorbance of standard nitrites. The mixture without the sample extract was used as control and compared with that of the Ascorbic acid as standard.

The Percentage of Nitric oxide free radical scavenging activity was calculated by the following formula:

$$\text{Percentage Nitric oxide free radical inhibition (\%)} = [(A_{\text{blank}} - A_{\text{sample}}) / (A_{\text{blank}} - A_{\text{control}})] \times 100$$

Where A_{blank} , A_{sample} and A_{control} are the absorbance of blank, sample and control, respectively. The half maximal inhibitory concentration (IC_{50}) was calculated from the Prism (GraphPad 5.0, Dose-response - Inhibition) curve obtained by plotting the percentage inhibition against the log concentration.

Anticholinesterasic activity assays

AChE assay was assessed by using the colorimetric method as explained by Ellman *et al.*, with slight modifications acclimatized for the enzymatic activity in supernatants of mice brain.²¹

In vitro inhibition of AChE enzyme

AChE inhibition of MCFE was considered by the proposed method by Ellman *et al.* with slight modifications.²¹ AChE activity was evaluated using the Acetylthiocholine iodide as substrate and 5, 5 - Dithiobis [2-nitrobenzoic acid] as a chromogen. The mice brain homogenate was sourced as acetylcholinesterase enzyme. Male mice of Swiss albino strain of about 20g body weight sacrificed by exerting cervical dislocation. The brain was excised immediately and homogenized using 0.1 mM sodium phosphate buffer (pH 7.0) and stored at -80°C till the use. MCFE (50-800 µg/ml) mixed in 2.6ml of 0.1mM sodium phosphate buffer (pH 7.0) and 5 µl of brain homogenate containing crude enzyme. The mixture was incubated for 5 min at 25°C. The reaction was initiated by adding 20 µl of Acetylthiocholine iodide. The inhibition of AChE was measured at 412 nm; the yellow colour formation indicates the hydrolysis of acetylthiocholine into 2-nitro-5-sulfidobenzene carboxylate anion due to the action of DTNB with thiocholine for 10 min.

The Percentage of AChE inhibitory activity was calculated by the following formula:

$$\text{Percentage AChE inhibition (\%)} = [(A_{\text{blank}} - A_{\text{sample}}) / (A_{\text{blank}} - A_{\text{control}})] \times 100$$

Where A_{blank} , A_{sample} and A_{control} are the absorbance of blank, sample and control, respectively. The half maximal inhibitory concentration (IC_{50}) was calculated from the Prism (GraphPad 5.0, Dose-response - Inhibition) curve obtained by plotting the percentage inhibition against the log concentration.

Ex vivo inhibition of AChE enzyme

Six Swiss albino male mice were placed in each group and treated with two different doses of MCFE (100 and 200 mg/Kg, p.o) and Neostigmine (0.5 mg/Kg, i.p) for a period of 15 days. The animals in control group received the same volume of 5 % CMC (administered with vehicle). On 15th day, the mice were decapitated using cervical dislocation method. The whole brain was dissected out quickly in an ice- cold plate and

homogenized using 10 volumes of ice cold 20 mM phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 rpm for 10 min. The supernatant was sourced for acetylcholinesterase enzyme estimation. The total AChE activity was measured by the method proposed by Ellman *et al.* (1961). Homogenate was mixed with a buffered solution containing Ellman's reagent (10 mM DTNB) and Acetylthiocholine iodide (0.8 mM). Rate of Hydrolysis was measured at 415 nm for 3 min with an interval of 30 sec, estimated by the formation of thiolate dianion of DTNB. The percentage of inhibition was calculated by comparing the enzymatic activity of AChE in brain homogenate aliquots with mice treated with 5% CMC. The Percentage of inhibition of AChE activity was calculated by using the following formula:

$$\text{Percentage AChE inhibition (\%)} = [(A_{\text{blank}} - A_{\text{sample}}) / (A_{\text{blank}} - A_{\text{control}})] \times 100$$

Where A_{blank} , A_{sample} and A_{control} are the absorbance of blank, sample and control, respectively. The half maximal inhibitory concentration (IC_{50}) was calculated from the Prism (Graph Pad 5.0, Dose-response - Inhibition) curve obtained by plotting the percentage inhibition versus the log concentration.

Statistical analysis

All activities were carried out in triplicate and the data were expressed as mean ± standard error mean (S.E.M.). The inhibitory concentration of free radicals by 50%, (IC_{50}) was graphically evaluated by a non linear regression method using Graph Pad Prism (Ver. 5.0) software. ANOVA followed by Duncan's multiple range tests was used to compare the treatments and control groups. Dose response relationship was obtained by linear regression. 95% ($p < 0.05$) confidence level were considered as statistically significant.

RESULTS & DISCUSSION

In vitro antioxidant assay

The results of the DPPH and nitric oxide free radical scavenging activity were reported in Table 1 and the IC_{50} values obtained are shown in Figure 1 & 2. It is very clear from the Table that the standard for DPPH free radical scavenging activity quercetin produced maximum percentage inhibition of 88.12±1.04 as compared to that of the MCFE which produced an inhibition of 76.77 ± 1.10. The standard ascorbic acid produced a maximum percentage inhibition of the nitric oxide free radicals of 94.75±0.36 at a dose of 800 µg/ml as compared to that of the MCFE which produced an inhibition of 71.74±0.60. Further all the standards and MCFE produced a dose dependent inhibition of DPPH and nitric oxide free radicals. This is evident from Figure 1 which was used to determine the IC_{50} value. The IC_{50} value of quercetin was found to be 46.22 µg/ml as compared to the MCFE which has an IC_{50} value of 43.14 µg/ml for DPPH free radical scavenging activity [Figure 2]. Similarly the IC_{50} value of ascorbic acid was found to be 81.85 µg/ml as compared to the MCFE which has an IC_{50} value of 148.0 µg/ml for nitric oxide scavenging activity.

In vitro inhibition of AChE enzyme

The values of *in vitro* anticholinesterase activity were tabulated in Table 2. The percentage inhibition values of MCFE were found to be in a dose dependent manner as represented in Figure 3. The maximum percentage inhibition was obtained at the dose of 800 µg/ml which produced a maximum inhibition of 76.77±1.10. Similarly the *in vitro* acetylcholinesterase inhibition assay of standard drug neostigmine was found to produce maximum inhibition of 88.12±1.04 at a dose of 800 µg/ml. The IC_{50} values of both MCFE and the standard drug were represented in Figure 3. The IC_{50} value of MCFE and neostigmine was found to be 31.84 µg/ml & 19.71µg/ml respectively and this is evident from the analysis that

Table 1: Inhibition profile of activities of fruit extract of *Morinda citrifolia* against nitric oxide production & DPPH radical scavenging activity as free radical scavengers

Treatment groups	Dose (µg/ml)	Nitric oxide production (% inhibition)	DPPH decolouration (% inhibition)
<i>Morinda citrifolia</i>	25	15.07±0.38	58.85±0.09
	50	17.50±2.15	62.51±0.05
	100	22.79±1.66	65.34±1.93
	200	26.26±1.51	68.65±0.41
	400	28.73±1.29	72.84±0.68
	800	71.74±0.60	76.77±1.10
Quercetin	25	-	52.12±0.72
	50	-	67.28±0.46
	100	-	75.98±0.95
	200	-	81.82±1.02
	400	-	84.54±1.24
	800	-	88.12±1.04
Ascorbic acid	25	73.28±0.90	-
	50	89.99±0.36	-
	100	91.01±0.78	-
	200	90.51±2.18*	-
	400	93.50±0.36*	-
	800	94.75±0.36*	-

All assays were performed in triplicate. Enzyme inhibition is expressed as percentage of control. Each value represents mean±S.E.M.

Table 2: *In vitro* Anticholinesterase activity of *Morinda citrifolia*

Treatment groups	Dose (µg/ml)	AChE inhibition (%)
<i>Morinda citrifolia</i>	25	58.85±0.09
	50	62.51±0.05
	100	65.34±1.93
	200	68.65±0.41
	400	72.84±0.68
	800	76.77±1.10
Neostigmine	25	52.12±0.72
	50	67.28±0.46
	100	75.98±0.95
	200	81.82±1.02
	400	84.54±1.24
	800	88.12±1.04

All assays were performed in triplicate. Enzyme inhibition is expressed as percentage of control. Each value represents mean±S.E.M.

Table 3: Effects of chronic administration of *Morinda citrifolia* (100 and 200 mg/kg p.o.) and neostigmine (0.5 mg/kg i.p.) on AChE activity in mice brain.

Treatment groups	Dose (mg/kg)	AChE inhibition (%)
Neostigmine	0.5	62.1 ±2.5*
<i>Morinda citrifolia</i>	100	37.8±2.1*
	200	46.8±2.9*

All assays were performed in triplicate. Enzyme inhibition is expressed as percentage of control (DMSO-treated mice). Each value represents mean±S.E.M. ANOVA followed by Duncan's test. * P < 0.05; significant from control.

is carried out using non linear regression analysis for plotting the line of best fit for the observed values in the Table 2 using statistical methods.

Ex vivo inhibition of AChE enzyme

The *ex vivo* AChE inhibition assay was tabulated in Table 3. The standard drug neostigmine showed a percentage inhibition of 62.1±2.5. The MCFE showed a percentage inhibition of 37.8±2.1 & 46.8±2.9 at a dose of 100 & 200 mg/kg respectively and this proved a dose dependent percentage inhibition of acetylcholinesterase activity. Further the data was analysed for significance of P value. The standard drug and MCFE treated group was compared with the control group. All the three treated groups showed a significant value of P < 0.05 as compared to that of control. This indicates that the activity of acetylcholinesterase enzyme has significantly decreased after treatment with the standard drug and MCFE.

The present study investigated the neuroprotective activity of *Morinda citrifolia* and it was identified by both *in vitro* and *ex vivo* techniques that the phytoconstituents in the plant has the ability to improve the learning and memory function by inhibiting the acetylcholinesterase. Further the antioxidant potential of the plant was also evident from the DPPH and nitric oxide scavenging activity. Future studies may be designed for chronic administration of *Morinda citrifolia* to further investigate the effect on *in vivo* experimentation and also to identify the safety and efficacy parameters at both preclinical and clinical stages.

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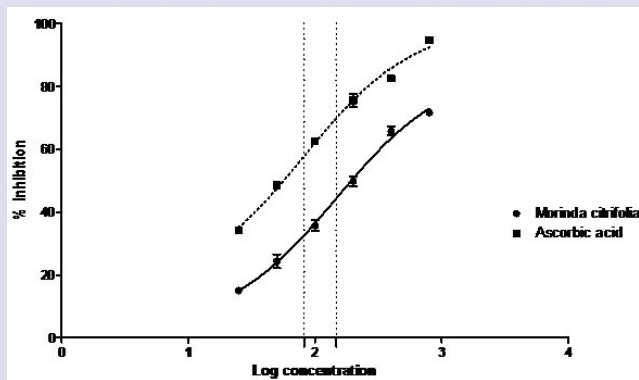


Figure 1: Nitric oxide scavenging assay of *Morinda citrifolia* fruit extract

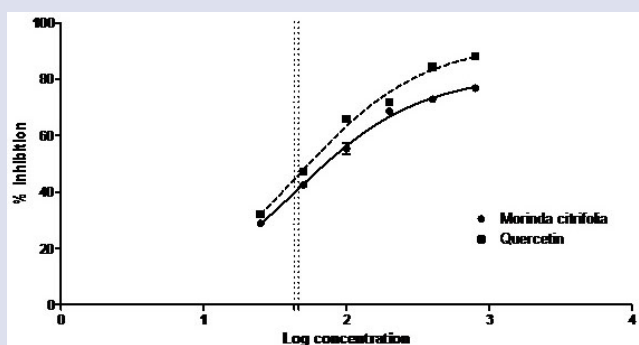


Figure 2: DPPH scavenging assay of *Morinda citrifolia* fruit extract

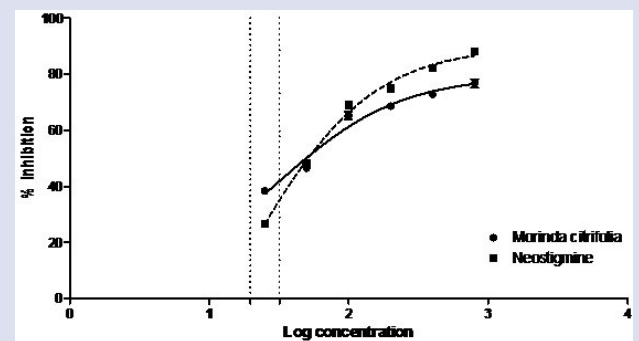


Figure 3: In vitro anticholinesterase activity of *Morinda citrifolia*

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Conflicting Interest (If present, give more details): Nil

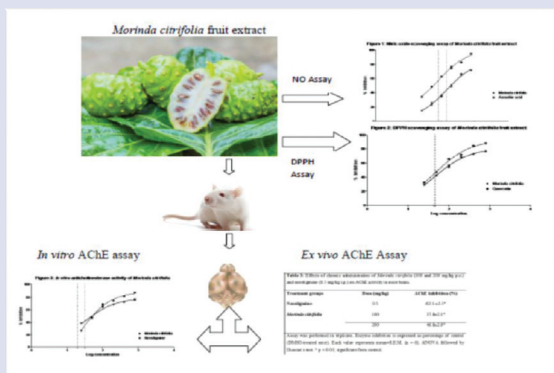
ABBREVIATIONS USED

MCFE: *Morinda citrifolia* fruit extract; AChE: Acetylcholinesterase; IC_{50} : Half maximal inhibitory concentration; DPPH: 1, 1-diphenyl-2-picrylhydrazyl radical; NO: Nitric oxide.

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GRAPHICAL ABSTRACT



ABOUT AUTHORS



Mr. J. Srikanth is working as Assistant professor in the Department of Pharmacology, Faculty of Pharmacy, Sri Ramachandra University and has got 8 years of teaching & research experience. He is currently pursuing Ph.D at Sri Ramachandra University. He has been awarded "Career Award for Young Teachers" (CAYT) - 2015 from All India Council for Technical Education (AICTE) for his Ph.D research proposal. He has also received "Growth and Advancement Towards Excellence" (GATE) – 2013 research project grant for young faculty from Sri Ramachandra University. His research area of interest includes Neuropharmacological screening of crude extracts, herbal & polyherbal formulations, In-silico docking analysis, Alternative to animal testing. He is a life member of APTI, LASA & SNCI.

SUMMARY

- The present study is focused to identify the neuroprotective activity of *Morinda citrifolia* fruit extract (MCFE) on *in vitro* and *ex vivo* animal model by inhibition of acetylcholinesterase (AChE), an enzyme target used for the treatment of Alzheimer's disease.
- Acetylcholinesterase inhibition assay was performed by *in vitro* method and the IC₅₀ value of MCFE and neostigmine was found to be 31.84 µg/ml & 19.71 µg/ml respectively.
- In *ex vivo* AChE inhibition assay the standard drug neostigmine showed a percentage inhibition of 62.1±2.5. The MCFE showed a percentage inhibition of 37.8±2.1 & 46.8±2.9 at a dose of 100 & 200 mg/kg body weight of the animal respectively and this proved a dose dependent percentage inhibition of acetylcholinesterase activity.
- The present study investigated the neuroprotective activity of MCFE and it was identified by both *in vitro* and *ex vivo* techniques that the phytoconstituents has the ability to improve the learning and memory function by inhibiting the acetylcholinesterase.

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