WWJMRD 2023; 9(07): 82-88
www.wwjmrd.com
International Journal
Peer Reviewed Journal
Refereed Journal
Indexed Journal
Impact Factor SJIF 2017: 5.182 2018: 5.51, (ISI) 2020-2021: 1.361
E-ISSN: 2454-6615

Riyama Shirin V. K
Department of Pharmacognosy and Phytochemistry, College of Pharmaceutical Sciences, Government Medical College, Kannur, Kerala University of Health Sciences, Kerala, India.

Dr. Arthi I
Department of Pharmacognosy and Phytochemistry, College of Pharmaceutical Sciences, Government Medical College, Kannur, Kerala University of Health Sciences, Kerala, India.

Correspondence:
Riyama Shirin V. K
Department of Pharmacognosy and Phytochemistry, College of Pharmaceutical Sciences, Government Medical College, Kannur, Kerala University of Health Sciences, Kerala, India.

In-Vitro Evaluation of Anti- Alzheimer Activity of *Alternanthera brasiliana* Leaf Extract

Riyama Shirin V. K, Dr. Arthi. I

Abstract
Being a medicinal plant, *Alternanthera brasiliana* plant also known as Brazilian joyweed is known for the treatment of various ailments in traditional medicine. The current study was carried out to analyse the anti-Alzheimer's potential of ethanolic extract of *Alternanthera brasiliana*. For this inhibition of beta amyloid induced neurodegeneration was determined by MTT Assay and acetylcholinesterase inhibition potential of *Alternanthera brasiliana* leaf was evaluated. Anti-lipid peroxidative effect of the extract was also determined by lipid peroxidation cell line assay. The plant has shown neuroprotective effect, significant acetylcholinesterase inhibitory activity and good antioxidant activity. Thus, plant offers to be important source of bioactive constituents for neurodegenerative disease.

Keywords: *Alternanthera brasiliana*, Neurodegeneration, Anti-Alzheimer effect, traditional medicine.

1. Introduction
Alzheimer's disease (AD) is an irreversible, progressive neurodegenerative disease that slowly destroys memory and thinking skills, eventually even the ability to carry out simplest tasks. It is the most common cause of dementia, that is difficulties with memory, language, problem solving and other cognitive skills. As per WHO, more than 55 million people live with dementia worldwide, over 60% of whom live in low- and middle-income families [1]. Allopathic medicines failed to provide an appropriate therapy there is no cure but current medications and management strategies may improve symptoms. So phytopharmacological screening of new drugs is an important strategy. The leaf extract showed neuropharmacological action, that is it possess stimulatory action in the CNS, and has anxiolytic activity [2]. It also possesses anti-inflammatory and antioxidant properties [3]. Flavonoids and phenols are also present [4]. This is the rationale for selecting this particular plant species for screening of anti-Alzheimer effects.

2.Materials and Method
2.1. Preparation of extract
Soxhlet Extraction: The powdered plant material (50g) was extracted by hot continuous extraction (soxhlet) method. The plant material was extracted with Ethanol (70% v/v) (500ml) for four days in a soxhlet apparatus. Initially 50g of the dried powdered leaves was weighed and transferred into a thimble for packing, wherein the content was wetted with ethanol and poured until the siphon tube was filled. A piece of porcelain was added to avoid bumping effect. The extractor was assembled and extracted at about 70-75°C until the colour of the solution in the siphon tube turns pale. The extracts obtained were dried at room temperature and the yield stored in desiccator.

2.2. Evaluation of Anti-Alzheimer Activity
2.2.1: Acetylcholinesterase (AChE) inhibition assay: [5,6]
Inhibition of AChE was determined according to the colorimetric method of Ellman *et al.* Ellman's method using an alternative substrate acetylthiocholine and 5, 5'-dithio-bis 2 nitrobenzoic acid (DTNB). The reaction results in production of 5-thio-2-nitrobenzoate that

has yellow colour due to the shift of electrons to the Sulphur atom. Reagents used are 0.1M phosphate buffer (pH 8.0) -5 ml, DTNB solution (0.4mg/ml), Enzyme solution and ATC (1mM acetylthiocholine)

Test tubes were taken and labelled as test and control. Added 2.4 ml of 0.1 M phosphate buffer (pH 8.0) to all the tubes. Then 0.1 mL of DTNB was added to the test tubes, after that added 0.2 mL of the enzyme solution (Acetylcholinesterase). To the test, added test sample and for control, solvent was added. Both control and each of the test solutions were preincubated at 25 °C for 5 min. After preincubation, the enzyme reaction was started by the addition of 40 µL of ATC. Further, incubated at 25 °C for 20 min. The absorbance at 412 nm was measured spectrophotometrically and compared with that of the control. Donepezil is used as the standard drug. Equation (1)

$$\% \text{ inhibition} = 1 - \frac{\text{Change in sample absorbance}}{\text{Change in blank absorbance}} \times 100$$

2.2.2: In-vitro Neuroprotective Effect Determination by MTT Assay [7,8]

Table 1: Instruments and reagents used.

DMEM media	Sigma Aldrich, USA D5648
Fetal Bovine Serum	Gibco, US origin-
0.25% Trypsin	Invitrogen, USA 25200-056
Micropipettes	F1 Thermoscientific USA
CO2 Incubator	Eppendorf, GERMANY
Phase Contrast Microscope	Olympus, JAPAN with Optika Pro 5 Camera
MTT	Sigma Aldrich M5655
ELISA Reader	ERBA, GERMANY
Culture Plates and Flasks	NUNC, Thermoscientific USA
Image Magnification	10 X

SHSY-5Y (Neuroblastoma cells) cell line was purchased from NCCS Pune was maintained in Dulbecco's modified eagles' media (HIMEDIA) from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's Modified Eagles medium (DMEM) (Sigma Aldrich, USA).

The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphoteracin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO2 incubator (NBS Eppendorf, Germany).

The viability of cells was evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cells seeding in 96 well plate

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO2 incubator.

Preparation of compound stock

1mg of the sample was weighed and completely dissolved in 1mL DMEM using a cyclomixer. The extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility. β amyloid (10µM) was added to induce toxicity.

Cytotoxicity Evaluation

After attaining sufficient growth, β amyloid(10µM) was added to induce toxicity and incubated for one-hour, prepared extracts in 5% DMEM were five times serially diluted by two-fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of 5% DMEM) and each concentration of100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO2 incubator.

Cytotoxicity Assay by Direct Microscopic observation

Entire plate was observed at an interval of each 24 hours; up to 72 hours in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity Assay by MTT Method

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization.

After 24 hours of incubation period, the sample content in wells were removed and 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution DMSO was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm (Laura B. Talarico et al., 2004)

The percentage of growth inhibition was calculated using the formula; Equation (2)

$$\% \text{ of viability} = \frac{\text{Mean OD Samples x 100}}{\text{Mean OD of control group}}$$

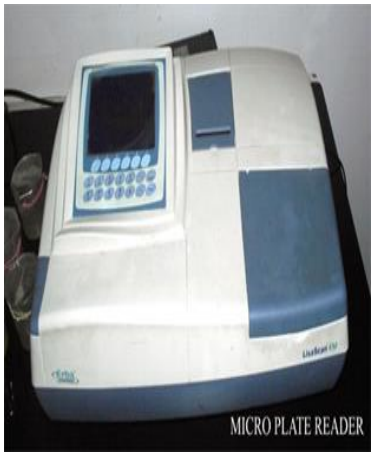

Fig. 1: Microplate reader

Fig. 2: Phase contrast microscope

Fig.3: CO2 Incubator

2.2.3 Lipid Peroxidation Cell Line Assay

Lipid peroxidation referred as the oxidative degradation of lipids. It was the process whereby free radicals "steal" electrons from the lipids in the cell membrane resulting in cell damage. This process proceeded by a free radical often affected poly unsaturated fatty acids because they contain multiple double bonds in between which lies methylene-CH2 groups that poses especially reactive hydrolysis. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) upon decomposition.

The cells were grown to 60% confluency, incubated with β amyloid (10µM) for one hour. After incubation the cells were exposed with sample (25µg/mL). Untreated well and β amyloid induced wells were also maintained and incubated for 24 hours. After treatment, treated cells were trypsinized with Trypsin-EDTA Solution (HiMedia), collected and transferred into eppendorf tubes. The tubes were centrifuged at 5000pm for five minutes. The supernatant was discarded and the pellet was suspended in 200µL of lysis buffer (0.1M tris, 0.2M EDTA, 2M NaCl, 0.5% Triton). The samples were incubated at 4^0C for 20 minutes. The cell lysate thus obtained was used for the study.

50µl of cell lysate were added with 500µl of 70% alcohol and 1ml of 1% TBA. All the tubes were kept in boiling water bath for 20 minutes. After cooling to room temperature added 50 µl of acetone to all the test tubes. Read the absorbance at 535 nm in spectrophotometer.

3.Results and Discussion

3.1: Acetylcholinesterase Inhibition Assay (Ellman's method)

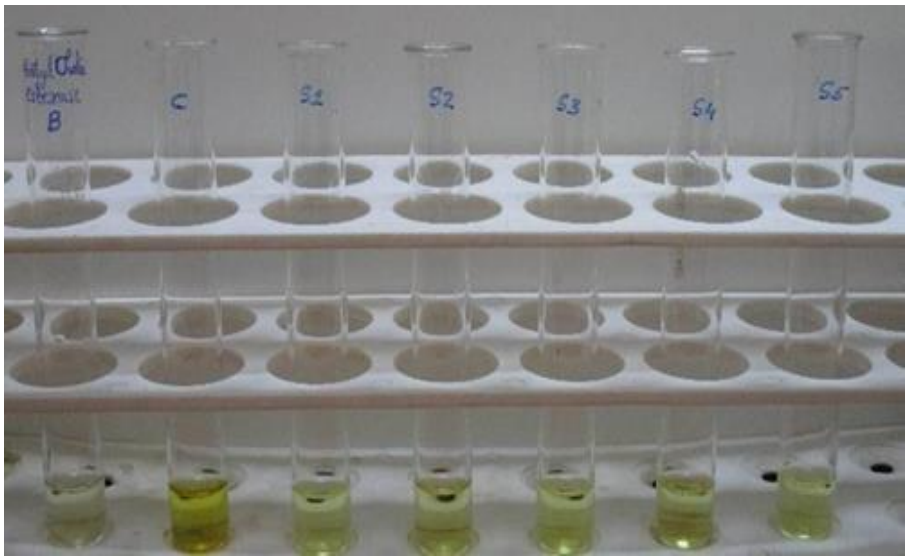

Fig. 4: Standard drug solution of varying concentration.

Table 2: Acetylcholinesterase inhibition assay performed on standard drug.

Sample	Concentration (µg)	OD at 412 nm		% of Inhibition
		INITIAL	FINAL	
Control	-	0.315	0.491	-
Donepezil	1.56	0.232	0.35	32.95
	3.12	0.32	0.4	54.55
	6.25	0.32	0.38	65.91
	12.5	0.41	0.42	94.32
	25	0.408	0.41	98.86

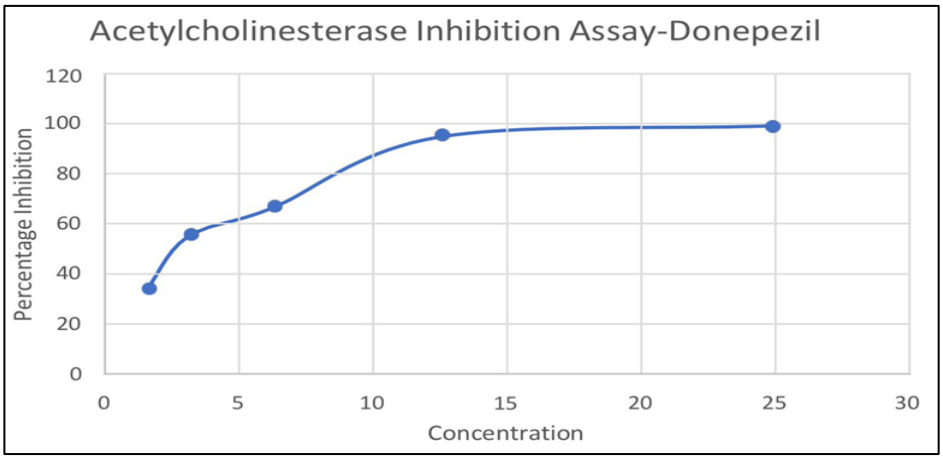

Fig. 5: Graphical representation of acetylcholinesterase inhibition by donepezil.

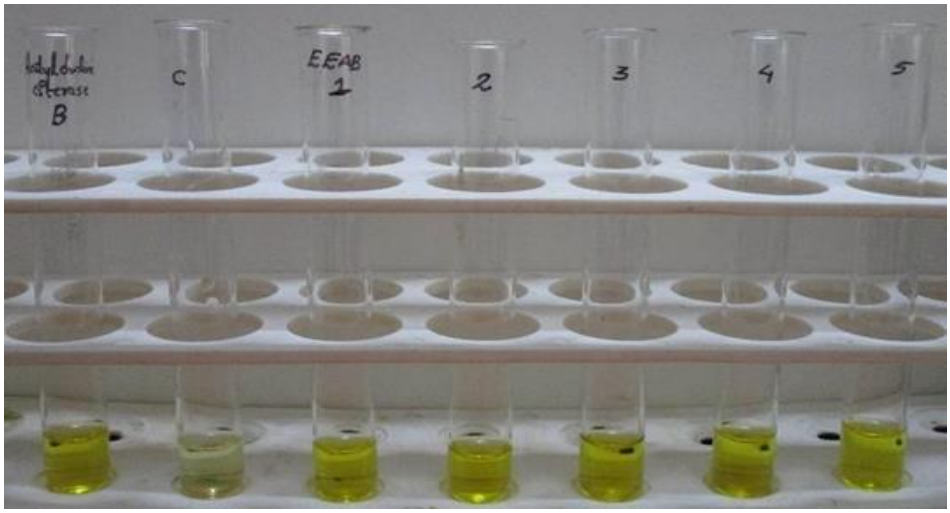

Fig. 6: Sample drug solution of varying concentration

Table 3: Acetylcholinesterase inhibition assay performed on sample drug

Sample	Concentration (µg)	OD at 412 nm		% of Inhibition
		INITIAL	FINAL	
Control	-	0.3000	0.3810	-
EEAB	6.25	0.2014	0.2749	9.26
	12.5	0.2440	0.2980	33.34
	25	0.2532	0.2765	71.24
	50	0.2140	0.2230	88.9
	100	0.2809	0.2868	92.71

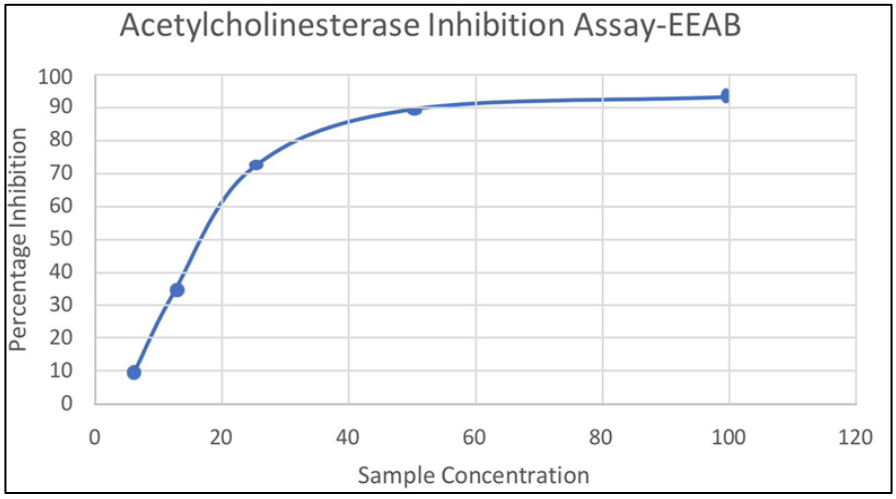

Figure 7: Graphical representation of acetylcholinesterase inhibition by sample drug.

Acetylcholinesterase (AChE) is a key enzyme in the cholinergic nervous system.[9] Therapies designed to reverse the cholinergic deficit in Alzheimer's disease is mostly AChE inhibitors, which enhance cholinergic transmission with transient therapeutic effects. Various studies have shown that cholinesterase inhibitors act on multiple therapeutic targets such as prevention of the formation of β-amyloid plaques, antioxidant activity and modulation of APP processing. However, there is a need for new AChE inhibitor lead compounds with much lower toxicity and higher central nervous system (CNS) penetration. In the present study ethanolic extract of *Alternanthera brasiliana* (EEAB) showed prominent result in acetylcholinesterase inhibition assay in a dose dependent manner.

AChE Inhibitory activity of different concentration of EEAB (6.25,12.5,25,50,100µg) was found to be 9.26,33.34,71.24,88.9 and 92.71% respectively. Here table no.3 shows percentage inhibition of AChE which is increasing with increasing concentration. The percentage inhibition of 100µg of EEAB was found to be 92.71. So, there is a significant increase in the percentage of inhibition of AChE from 9.26 to 92.71, when compared with the percentage inhibition of standard drug donepezil. EEAB is showing significant increase in percentage inhibition with increasing concentration. The percentage inhibition of standard drug was found to be 32.9, 54.5, 65.9 ,94.3, 98.8 for 1.56,3.12,6.25,12.5,25µg respectively.

3.2: Evaluation of Neuroprotective effect by MTT Assay

The percentage of growth inhibition was calculated using the formula; Equation (3)

$$\% \text{ of viability} = \frac{\text{Mean OD Samples x 100}}{\text{Mean OD of control group}}$$

Table 4: Determination of Neuroprotective effect of sample drug by MTT assay.

Sample Concentration (µg/ml)	OD I	OD II	OD111	Average Absorbance @ 540nm	Percentage Viability
CONTROL	0.976	0.9783	0.9625	0.9723	100.00
Amyloid	0.5011	0.4924	0.4917	0.4951	50.92
6.25	0.6238	0.6342	0.6216	0.6265	64.44
12.5	0.8307	0.8327	0.8314	0.8316	85.53
25	0.9071	0.9082	0.9052	0.9068	93.27
50	0.7874	0.7842	0.7863	0.7860	80.84
100	0.7622	0.7631	0.7625	0.7626	78.43

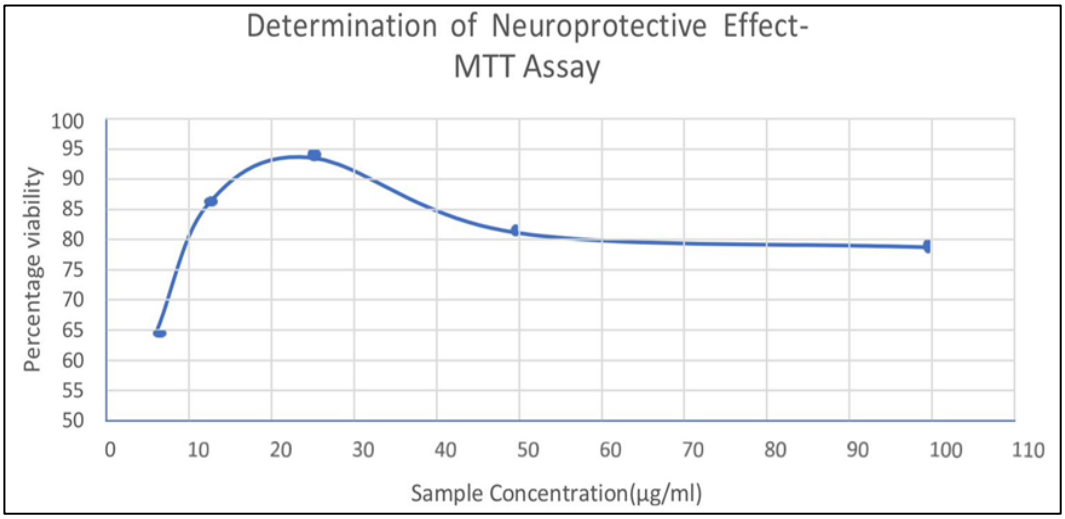

Fig. 8: Graphical representation of neuroprotective effect exhibited by sample drug.

One of the hallmarks in the development of AD is the aggregation of amyloid protein fragments in the brain, and much evidence points towards beta-amyloid fragments being one of the main causes of the neurodegenerative processes.[10]

All experiments were done in triplicates and average taken. The neuroprotective effect of EEAB at concentration 6.25,12.5 and 25,50,100µg/ml on β amyloid induced cytotoxic cells was measured using MTT Assay by studying variations of the viability of cells. The above figures showed that after exposure to 10µM β-Amyloid for 1 hour, the cell viability decreases to 50.92% compared to control which was 100%. However, its cytotoxicity was affected by varying concentration of EEAB. The cell survival was restored to 64.4,85.5,93.2,80.8,78.4 for 6.25,12.5,25,50,100µg/ml concentration thus exhibiting maximum percentage viability at 25µg/ml.

The drug protected neurons against Amyloid beta toxicity, and drug treated cultures exposed to Amyloid beta showed enhanced survival over Amyloid beta-only cultures for several days.

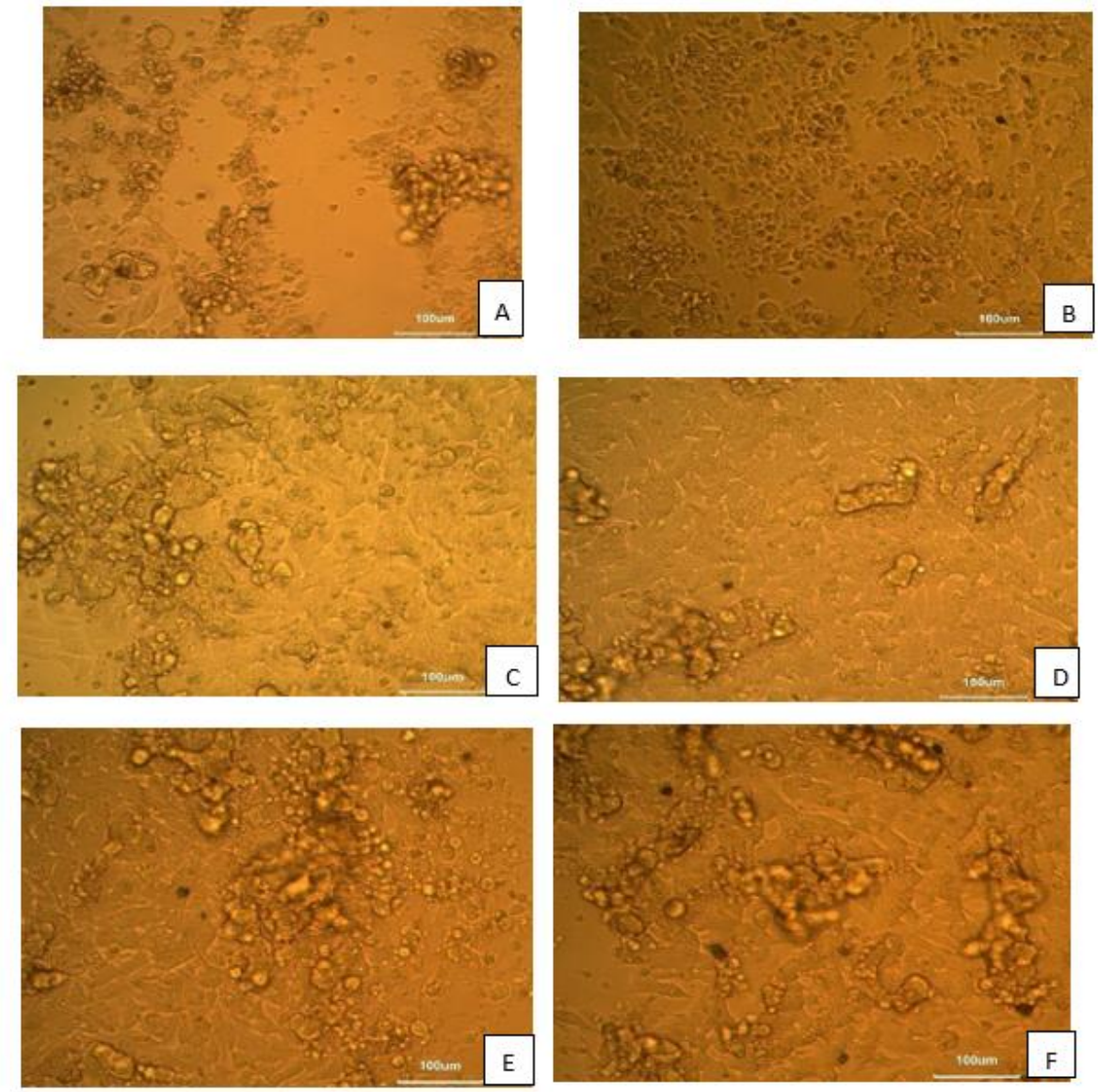

Fig. 9: Figures representing percentage viability at various concentrations: A) Amyloid, B) 6.25µg/ml, C) 12.5µg/ml, D) 25µg/ml, E)50µg/ml, F) 100µg/ml

3.3: Lipid Peroxidation Cell Line Assay

Table 5: Determination of Anti-lipid peroxidative effect of sample.

Sample	Absorbance	µg/MDA units
control	0.057	0.43149
amyloid	0.1258	0.952306
A+S	0.0908	0.687356

The values indicate that the extract has significant anti-lipid peroxidative effect as the amount of malondialdehyde generated has lowered upon addition of the sample to the amyloid induced cells.

There is substantial evidence that oxidative stress is a causative or at least ancillary factor in the pathogenesis of major neurodegenerative diseases [11]. Increased lipid peroxidation and oxidation of DNA as well as proteins are found in the substantia nigra patients. Nervous tissue has high lipid content and high aerobic metabolic activity, both make it susceptible to oxidative damage. Since oxidative damage is a major problem in neurodegenerative diseases, anti- lipid peroxidative effect has drug therapy has a vital role in the treatment of disease.

Conclusion

The results obtained suggests that the ethanolic extract of *Alternanthera brasiliana* has good acetylcholinesterase inhibitory effect, significant neuroprotective activity and antioxidant activity and hence studies confirms that the constituents of *Alternanthera brasiliana* have a promising effect on neurodegeneration and further studies have to be conducted to explore and confirm the possible mechanism of action.

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