



**IN-VITRO EVALUATION OF ANTI-ALZHEIMER EFFECTS ON WHOLE PLANT OF
DESMODIUM TRIFLORUM (L.) DC**

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ABSTRACT

Desmodium triflorum (L.) DC (Fabaceae) is a perennial medicinal plant which has a long history of use in traditional medicine for various treatment. The present study evaluates the pharmacognostic and pharmacological potential of methanolic extract of *D. triflorum* against *in vitro* model of Alzheimer's disease. Pharmacognostic evaluation including examination of morphological characters, microscopic characters, ash value, powder analysis, and extractive values were carried out. Phytochemical screening including chemical examination were also carried out. Anti-Alzheimer's activity of *D. triflorum* were evaluated using acetylcholinesterase inhibition cell line assay and beta amyloid neurodegeneration cell line assay in PC12 cells. The Phytochemical investigation of *D. triflorum* revealed the presence of various important secondary metabolites such as carbohydrates, proteins, amino acids, glycosides, flavonoids alkaloids and phenols in methanolic extract. AChE inhibitory activity of various concentrations of the extract (25, 50 and 100 µg/ml) showed significant increase in the percentage inhibition of AChE enzyme from 19.24 to 66.28, when compared with the percentage inhibition of standard drug Donepezil hydrochloride. Test sample at highest concentration (100 µg/ml) showed maximum inhibition on AChE activity as compared to standard Donepezil hydrochloride alone. In beta amyloid -induced cytotoxicity the cell viability was measured using MTT assay. Here *D. triflorum* methanolic extract at concentrations of 1.5, 3.1, 6.25, 12.5 and 25 µg/ml significantly restored cell survival to 53.44%, 59.80%, 67.78%, 75.75% and 89.34% respectively, exhibiting cytoprotection in a dose-dependent manner. The study concludes that *D. triflorum* possesses neuroprotective activity on PC12 cells with significant AChE inhibitory activity.

KEYWORDS: *Desmodium triflorum* (L.) DC; Anti-Alzheimer's activity; Acetylcholinesterase inhibition cell line assay; Beta amyloid neurodegeneration cell line assay; PC12 cells; MTT assay.

INTRODUCTION

Plants as herbal drugs or herbal medicines are in connection with mankind dates back to paleolithic age that is about 60,000 years ago. The use of herbal drugs as a complementary or alternative therapy in modern medicine has gained significant attention due to their low toxicity, cost-effectiveness, and availability.

Neurodegenerative disorders in humans are like always most challenged, serious and wide spread issue in modern medicines due to their complicated pathogenesis and researchers are in a serious act to find alternative therapies for their treatment, since the current therapeutics seems to be less effective. Herbal medicines are a promising therapeutic approach towards neurodegenerative disorders and scientists are exploring them with great effort. Herbal medicines can be used in neurodegenerative disorders due to their multi functional and multi target characteristics most probably due to the

presence of phytoconstituents that has already proven for various activities like antioxidant, anti-inflammatory, anticholinesterase etc which is relevant for neuroprotective action.^[1]

Alzheimer's disease is a progressive neurodegenerative disorder which affects older individuals and is the most common cause of dementia. It may progress to a totally vegetative state. Atrophy of cortical and subcortical areas of brain is associated with deposition of β -amyloid proteins in the form extracellular senile (amyloid) plaques and formation of intracellular neurofibrillary tangles. There is also marked cholinergic deficiency in the brain, glutamate and neuropeptide neurotransmitter systems also affected.^[2]

Desmodium triflorum (L.) DC is a well-known Sri Lankan perennial medicinal plant which is in folkloric use belonging to family Fabaceae/Leguminosae. Roots,

leaves and the whole plant are used in Ayurvedic medicine for various treatment purposes. It is native to tropical and subtropical regions of the world, including Asia, Africa, and Australia. It is a small shrub with leaves and stems that have been traditionally used in Ayurveda and other traditional medicine systems to treat various health conditions, including fever, digestive disorders, and respiratory problems.^[3,4]



Figure 1: *Desmodium triflorum* (L.) DC whole plant.

MATERIALS AND METHODS

Collection and plant Authentication of plant

Fresh whole plant of *Desmodium triflorum* (L.) DC was collected locally from Calicut, Kerala (India) in August, 2022. The plant was authenticated by Dr. Sreeja.P M.Sc., Ph.D., PG Dept. of Botany and Research, Sir Syed College, Thaliparamba, Kannur and a voucher specimen with Accession No: 9939 of the *Desmodium triflorum* was deposited for future reference. The collected plant was then shade dried and powdered using an electric blender.



Figure 2: Herbarium of *Desmodium triflorum* (L.) DC whole plant.

Pharmacognostical study^[5,6,7]

Macroscopic evaluation

Macroscopic studies were carried out using organoleptic evaluation method. The shape, size, color, odour, taste, fracture, surface of leaf, root and stem of plants were observed. Macroscopic and microscopic characters were studied as described in quality method.

Microscopical studies

a) Transverse section

The samples were soaked in water before sectioning. Transverse sections were taken and stained according to the standard protocols. Stains was prepared using safranin (0.5% in distilled water). The stained sections were washed with water, mounted on clean slide and covered with cover slip using glycerine, observed under the microscope and the required photographic images were captured (LEICA M 80 stereo microscope with camera lucida)

b) Powder analysis

Shade dried whole plant were finely powdered and studied under microscope. Small quantity of powder was placed on slide and mounted with 2-3 drops of safranin and slide was covered with cover slip then lignified structures were examined under microscope and photography was done by using digital camera.

Physicochemical characters

Physicochemical constants like ash value, extractive value, loss on drying, swelling index, foaming index and mucilage content were carried out according to the standard procedure

Phytochemical study

Methanolic extracts of whole plant of *Desmodium triflorum* were subjected to different chemical tests separately for the identification of various active constituents.

Preparation of methanolic *Desmodium triflorum* extract

The dried *Desmodium triflorum* powder kept for extraction purpose using methanol by maceration method. The plant was washed thoroughly with clean water, cut into small pieces, and shade dried for 6 weeks. Coarse powder of plant was made with a grinder. About 50g coarse dry powder of *Desmodium triflorum* whole plant were separately subjected to maceration process with 600 ml methanol at room temperature.

Pharmacological study

Determination of Acetylcholinesterase inhibitory activity using microplate assay based on Ellman's method.^[8,9]

Inhibition of AChE was determined according to the colorimetric method of Ellman *et al.* The principle of the method is the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolysed. This is accomplished by the continuous reaction of the thiol with

Ellman's reagent 5: 5-dithiobis-2-nitrobenzoate ion (DTNB) to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The rate of colour production is measured at 412 nm in a photometer.

50 mM Tris-HCl pH 8.0 was used as buffer throughout the experiment unless otherwise stated. AChE used in this assay was from *Electrophorus electricus* (electric eel) (Type VI-S lyophilised powder, 222 U/mg solid 268 U/mg protein). The enzyme stock solution (222 U/mL) was kept at -80°C. The further enzyme-dilution was done in 0.1% BSA in buffer. DNTB was dissolved in this buffer containing 0.1 M NaCl and 0.02 M MgCl₂. ATCI was dissolved in deionized water.

100µL of 3mM DNTB, 20µL of 0.26 U/mL of AChE and 40µL of buffer (50 mM Tris pH 8.0), 20µL of each extract in various concentrations (25, 50, 100, µg/mL) dissolved in buffer, was added to 96 well plate. After mixing, the plate was incubated for 15 minutes (25 °C) and then the absorbance was measured at 412nm. Then the reaction initiated by the addition of 20µL of ATCI. After mixing, absorbance was measured at two-time intervals (5minutes, 20 minutes).

$$\% \text{ Inhibition} = (E \times \Delta OD) / E \times 100$$

E = the activity of the enzyme without extract
OD = activity of enzyme with the extract

Invitro neuroprotective effect determination by MTT Assay.^[10,11]

PC12 (Neuroblastoma) 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 cm² tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37°C 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 (5x10⁴ cells/well) was seeded in 96 well tissue culture plate and incubated at 37°C in a humidified 5% CO₂ incubator.

Preparation of compound stock

1mg of the sample was weighed and completely

dissolved in 1mL 0.1% DMSO using a cyclomixer. The extract solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility. βamyloid(10µM) was added to induce toxicity.

Cytotoxicity Evaluation

After attaining sufficient growth. βamyloid(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 (25µg, 12.5µg, 6.25µg, 3.1µg, 1.5µg in 500µl of 5% DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37°C 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 30 µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated 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.

The percentage of growth inhibition was calculated using the formula

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

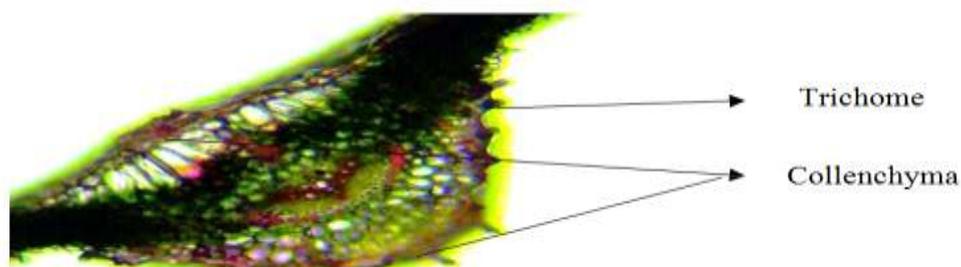
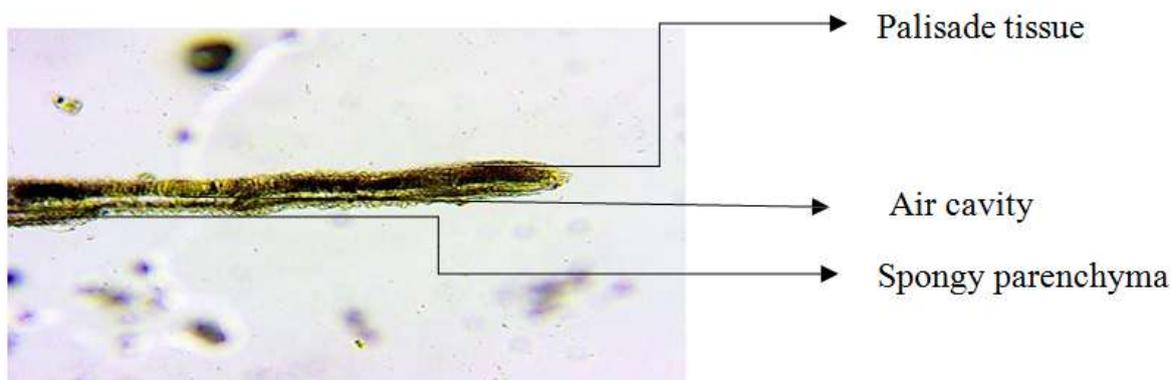
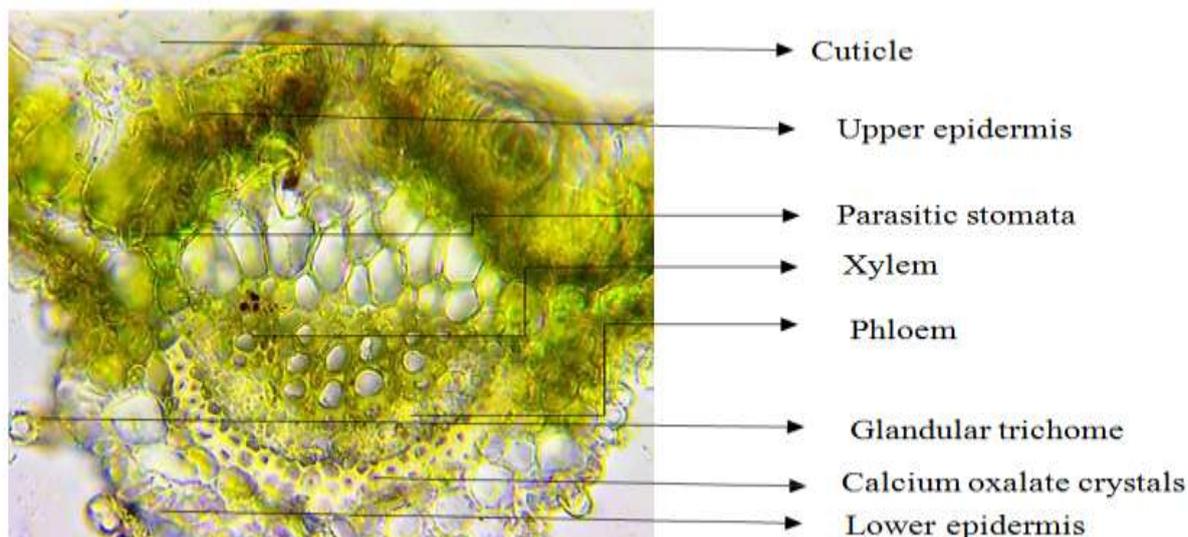
RESULTS AND DISCUSSION

Macroscopic evaluation

Macroscopical characters of *Desmodium triflorum* (L.) DC whole plant described in the table 1.

Table 1: Morphological and organoleptic characters of *Desmodium triflorum*.

Characters	Leaf	Stem	Root
Shape	Oval and acuminate	Cylindrical	Cylindrical
Size	1-2cm	15-45 cm	1-5mm (D)
Colour	Green	White or brownish yellow	White or brown
Taste	Characteristic	Characteristic	Astringent or mucilaginous
Odour	Characteristic	Characteristic	No characteristic odour
Fracture	-	Short	Soft
Surface	Glabrous and smooth	Glabrous or hairy	Rough due to presence of small lentils

Microscopic studies**Transverse section of leaf****Figure 3: Transverse section of leaf (10X).****Figure 4: Mesophyll part of leaf (10X).****Figure 5: Transverse section of midrib (40X).**

The T.S of leaf showed its typical dorsiventral nature. Upper and lower epidermis contain rectangular single layer covered with cuticle, lamina, mesophyll, and midrib region were observed as important diagnostic characters. Parasitic stomata are seen in upper layers. Palisade tissue appears in double layer just below upper epidermis in lamina region. Midrib shows central

nonlignified phloem, lignified xylem and parenchyma cells with calcium oxalate crystals. Well-defined patches of collenchyma were observed above and below the vascular bundles in the midrib area. Mesophyll region shows loosely arranged spongy parenchyma cells. Covering and glandular trichomes are present and mostly seen at lower epidermis.

Transverse section of stem

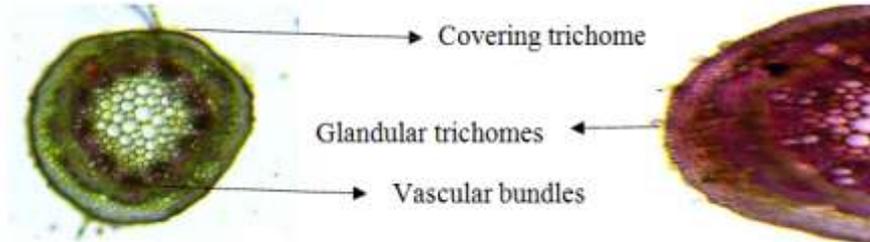


Figure 6: Transverse section of stem (10X).

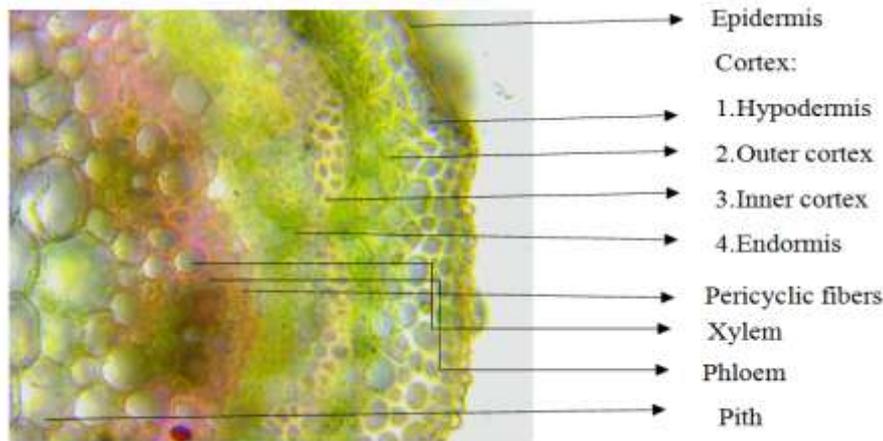


Figure 7: Transverse section of stem (40X).

The T.S of stem also shows presence of a single layered epidermis. Inner to epidermis is cortex that is distinct to 4 zones; hypodermis without chloroplast, outer cortex which is chlorenchymatous with plenty of intercellular space; inner cortex with loosely arranged parenchyma cells and endodermis that is innermost to cortex with compactly arranged parenchyma cells. Next to

endodermis, pericycle layer is present which forms a bundle sheath to the vascular bundles. About 10 to 11 conjoint vascular bundles is arranged as broken ring in ground tissue. Vascular bundles contain xylem and phloem. Inner to vascular bundles pith is present. The epidermis of stem also consists of covering and glandular trichomes.

Transverse section of root

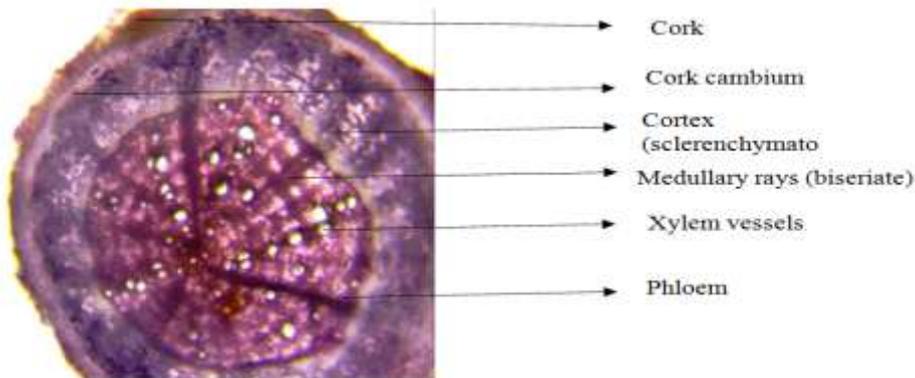


Figure 8: Transverse section of root (40X).

The T.S of root shows presence of outer layer of cork cells which is stratified and 3-6layered. Cork cambium is present and the cortex is 3-8 layered sclerenchymatous cells. Fibers are scattered. Phloem is secondary and 4-6 layered with sieve tubes, companion cells, fibres, and

phloem parenchyma. Xylem vessels is sclerenchymatous in scattered groups, cells filled with dark contents, parenchyma cells filled with starch and the medullary rays are 2-3 seriate filled with starch grains.

Powder microscopy

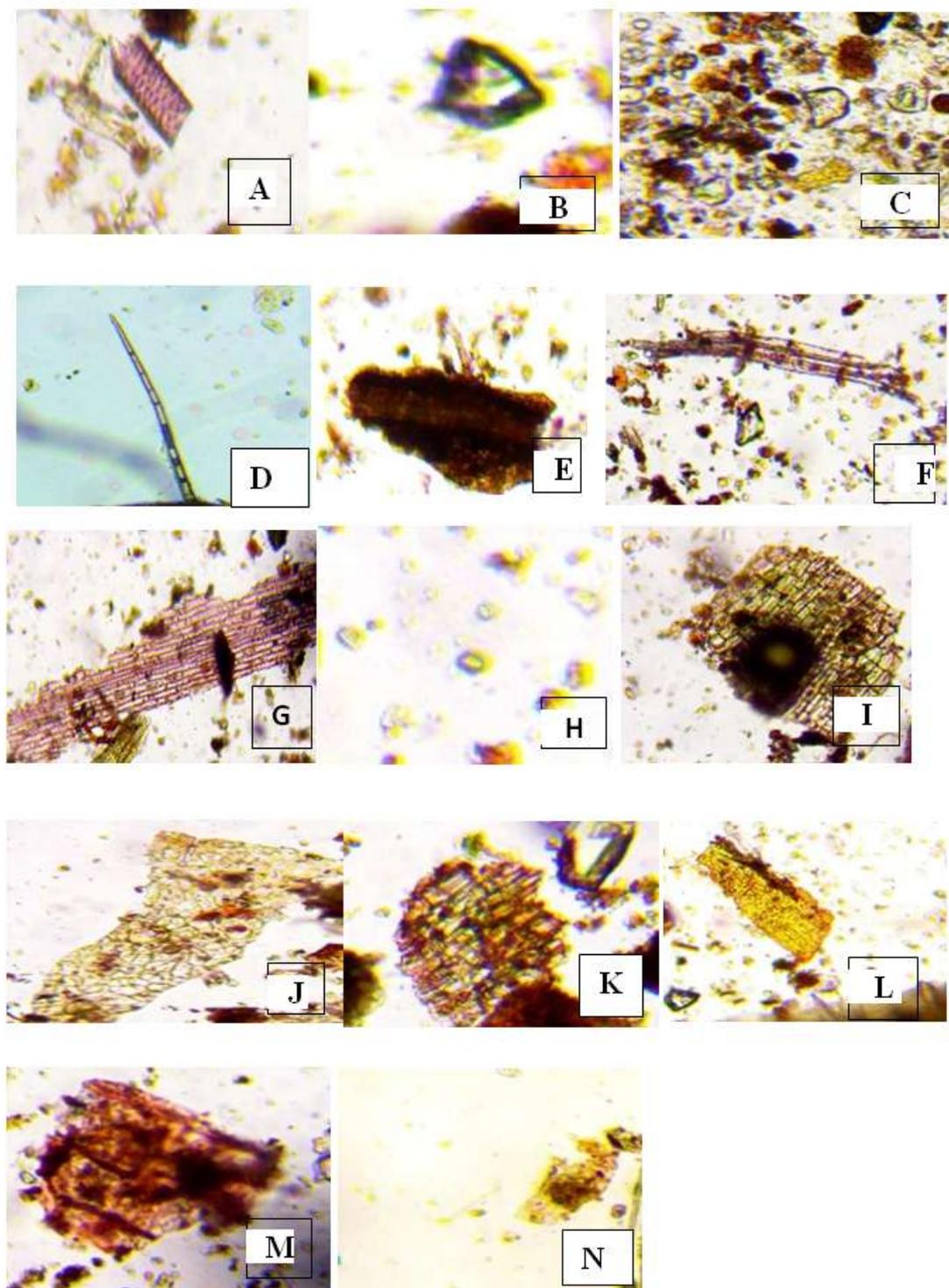


Figure 9: Powder microscopy, A- Pitted xylem vessels, B- Prismatic calcium oxalate crystals, C- Brown matters, calcium oxalates and stone cells, D-Trichomes, E-Epidermal cells with trichomes, F-Simple fibers, G- Fragment of lignified fiber, H- Starch grains, I- Lignified parenchyma cells, J-Stone cells in parenchyma, K- Cork cells, L- Wood element, M-Parenchyma, N-stomata.

1.2. Physicochemical Characters

Various physicochemical parameters were analysed for the confirmation of identity & purity. The extractive

values with alcohol and water, loss on drying, swelling and foaming index and mucilage content were also determined.

Table 2: Physicochemical parameters of *Desmodium triflorum*(L.)DC.

SL.NO	Physicochemical Parameters	Results
1	Ash value	(Percentage w/w)
a	Total ash	9.54
b	Acid insoluble ash	3.54
c	Water soluble ash	5.32
2	Extractive value	(Percentage w/w)
a	Alcohol soluble extractive	8.9
b	Water soluble extractive	18.8
3	Loss on drying	4.1 % w/w
4	Foaming index	Nil
5	Swelling index	6%
6	Mucilage content	Nil

Phytochemical study

A systematic preliminary phytochemical evaluation was carried out on the MEDT to explore chemical constituents of *D.triflorum* and the results indicates the presence of carbohydrates, proteins, amino acids,

steroids, glycosides, saponins, alkaloids, tannins, flavonoids and phenolic compounds. the colour of the extract was found to be dark greenish colour with sticky consistency. The percentage yield was found to be about 3.3 % w/w.

Qualitative chemical examination

Table 3: Phytoconstituents present in DTME.

S.No	Phytoconstituents	Methanolic extract
1	Carbohydrates	+
1a	Monosaccharides	-
1b	Free reducing sugar	-
1c	Combined reducing sugar	-
1d	Soluble starch	-
2	Proteins	+
3	Amino acids	+
4	Fats and oils	-
5	Steroids	+
6	Volatile oils	-
7	Glycosides	+
7a	Cardiac glycosides	+
7b	Anthraquinone glycosides	-
7c	Saponin glycosides	+
7d	Cyanogenetic glycosides	-
8	Phenolic compounds	+
9	Flavonoids	+
10	Alkaloids	+
11	Tannins	+
12	Terpenoids	-

(+) Presence of phytoconstituents (-)Absence of phytoconstituents



Figure 10: methanolic extract of *Desmodium triflorum* (L.) DC with darkgreenish colour and sticky consistency.

Pharmacological study

Determination of acetylcholinesterase (AChE) inhibitory activity using microplate assay.

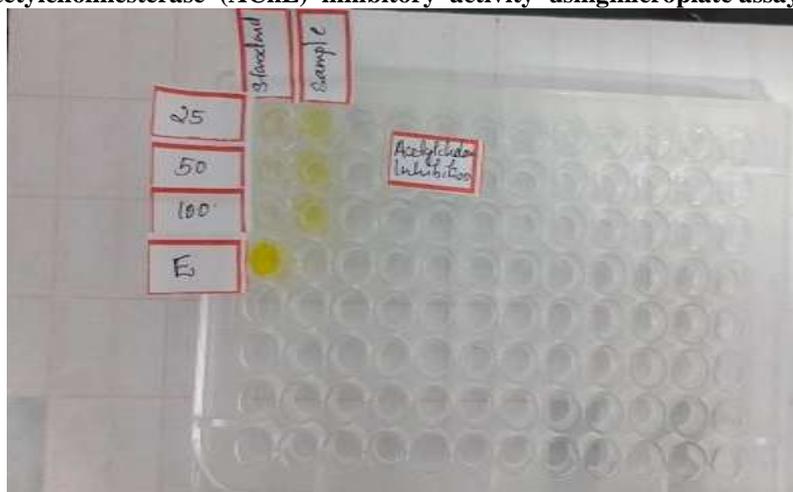


Figure 11: Standard and sample drug solution of varying concentration.

Table 4: Acetylcholinesterase inhibition assay performed on sample and standard drug

Conc.(µg/ml)	Optical density at 412 nm			% of inhibition		
	Control	Standard (donepezilHcl.)	Test	Control	Standard (donepezil Hcl.)	Test
25	0.1247	0.3665	0.1924	-	36.65	19.24
50		0.6913	0.3512		69.13	35.12
100		0.9031	0.6628		90.31	66.28

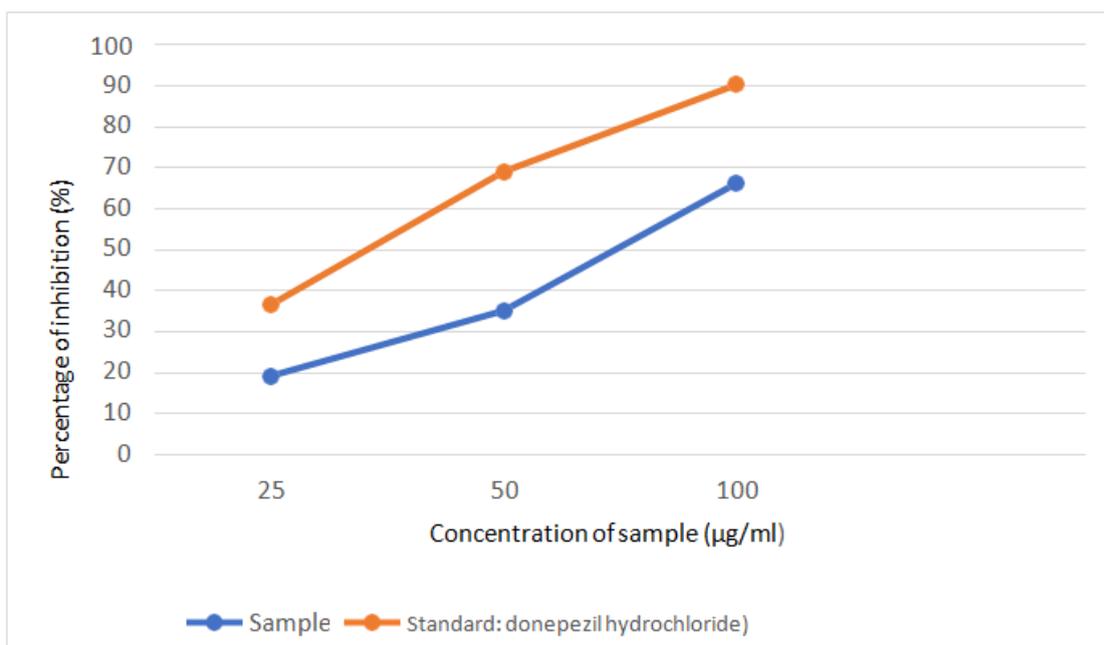


Figure 12: Graphical representation depicting acetylcholinesterase inhibitory activity of Sample and standard (Donepezil hydrochloride).

Table 5: Acetylcholinesterase IC50 of test and standard.

Sample	IC50 (µg/ml)
MEDT	73.99
Donepezil hydrochloride	35.27

AChE inhibitory activity of different concentration of MEDT (25,50 and 100 µg/ml) was found to be 19.24, 35.12 and 66.28 respectively. Here table no :7 shows percentage inhibition which is increasing with increase in concentration. The percentage inhibition of 100 µg/ml of MEDT was found to be 66.28. So, there is a significant increase in the percentage inhibition of AChE enzyme from 19.24 to 66.28, when compared with the percentage inhibition of standard drug donepezil hydrochloride. MEDT is showing a significant increase in percentage inhibition with increase in concentration. The percentage inhibition values of standard drug were found to be 36.65, 69.13 and 90.31 for 25, 50 and 100 µg/ml.

IC50 values (concentration of sample required to inhibit 50% of acetylcholinesterase enzyme) was calculated using ED50 PLUS V 1.0 Software. MEDT shows moderate inhibitory activity with IC50 73.99 µg/ml compared to the standard with IC50 35.27µg/ml.

Determination of *Invitro* neuroprotective effect by MTT assay

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD sample group}}{\text{Mean OD of control group}} \times 100$$

Table 6: Absorbance and percentage viability by MTT assay.

Concentration of MEDT(µg/ml)	OD I	OD II	OD III	Average Absorbanceat 540nm	Percentage Viability
1.5	0.193	0.2003	0.2045	0.1993	53.44
3.1	0.2148	0.2288	0.2254	0.2230	59.80
6.25	0.2442	0.2593	0.2547	0.2527	67.78
12.5	0.2759	0.2859	0.2856	0.2825	75.75
25	0.3352	0.3286	0.3356	0.3331	89.34
Control	0.3874	0.3529	0.3785	0.3729	100.0
β amyloid	0.1894	0.1728	0.1754	0.1792	48.06

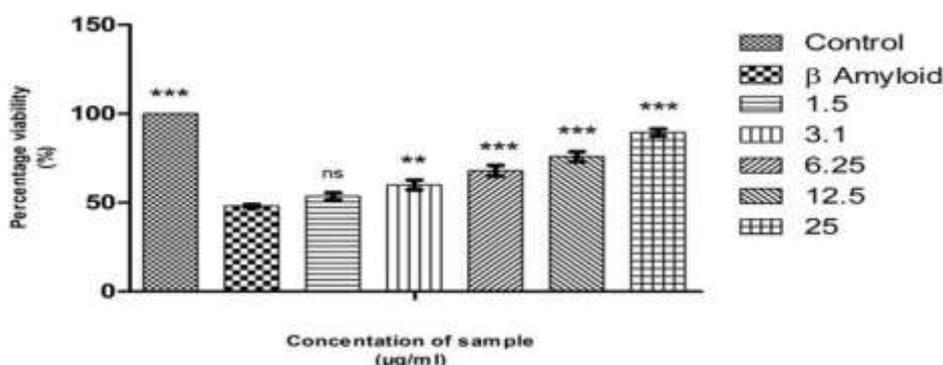


Figure 13: Bar graph depicting the neuroprotective effect of sample by MTT assay (X axis varied concentration of sample, Y axis Percentage viability).

All experiments were done in triplicates and results represented as Mean+/- SE. One- way ANOVA and Dunnett's test were performed to analyse data. ***p< 0.001

compared to β amyloid exposed group, **p< 0.01 compared to β amyloid exposed group, ns – non significant compared to β amyloid exposed group.

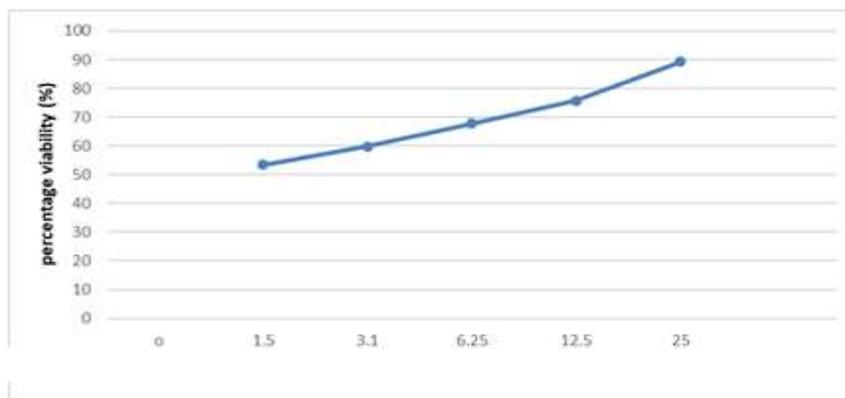


Figure 14: Line graph depicting the neuroprotective effect of sample by MTT assay.

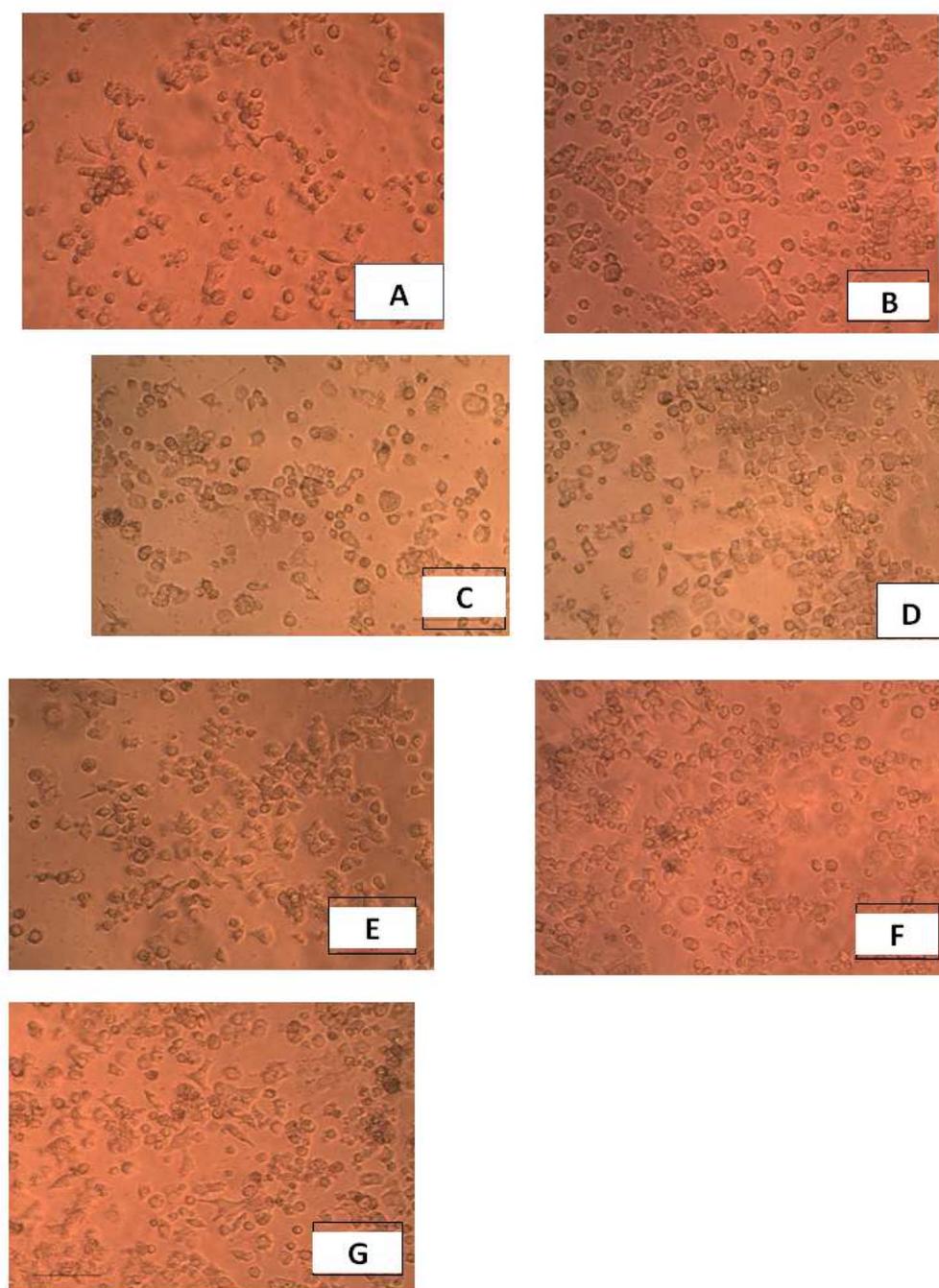


Figure 15: Viability of PC12 cells at various concentrations A) Beta amyloid B)Control, C) MEDT at 1.5µg/ml, D) MEDT at 3.1 µg/ml, E) MEDT at 6.25 µg/ml, F) MEDT at 12.5 µg/ml G) MEDT at 25 µg/ml

The neuroprotective effect of MEDT at concentrations 1.5,3.1,6.25,12.5 and 25 µg/ml on β amyloid induced cytotoxic PC12 cells was measured using MTT assay by studying the variations of viability of the cells. The above figures showed that after exposure to 10µM β -amyloid for 1h, the cell viability decline significantly to 48% compared to control which was 100%. However, its cytotoxicity was affected in the presence of different concentration of MEDT. The cell survival was restored to 53.44 %, 59.80%,67.78%,75.75% and 89.34% for 1.5,3.1,6.25,12.5 and 25 µg/ml respectively exhibiting cytoprotection in a dose-dependent manner.

CONCLUSION

So, in the current study, the data provide useful results to discover *D.triflorum* as a new source of lead compounds for the treatment of neurodegenerative disorders. The findings suggests that *D.triflorum* possesses neuroprotective activity on PC12 cells with significant AChE inhibitory activity. And the previous studies on *Desmodium triflorum* suggest that it contains phytoconstituents that confirms its neuroprotective activity and further studies are to be conducted to confirm the possible mechanisms of action and isolation and characterisation of novel isolated compounds.

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