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Research Article

Exploration of the Neuroprotective Potential of Averrhoa Bilimbi LEAF Extract USING an Invitro METHOD

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ABSTRACT

This project investigates the neuroprotective effects of macerated *Averrhoa bilimbi* (*A.bilimbi*) leaf. *A.bilimbi* is rich in bioactives such as flavonoids, phenols, alkaloids, tannins and terpenoids and coumarin etc. and has both antioxidant and neuroprotective effects. The neuroprotective effect of *A.bilimbi* macerate was investigated on SHSY5Y cell lines via the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Leaves of *A.bilimbi* were macerated and various concentrations of macerates prepared. These were then used to determine the neuroprotective abilities of the macerate in protecting the neuronal cells against a neurotoxicant, such as Hydrogen Peroxide (H_2O_2). The MTT assay was utilised to determine cell viability as this relates to mitochondrial function. Findings revealed that *A.bilimbi* leaf has a dose dependent effect in increasing cell viability and reducing H_2O_2 associated oxidative cell damage, hence demonstrating its neuroprotective effects. This research wishes to further explore the neuroprotective properties of macerated *A.bilimbi* leaves and how they may provide protection to neurons from oxidative stress.

INTRODUCTION

Averrhoa bilimbi Linn, commonly known as bilimbi, is a medicinal plant belonging to the family Oxalidaceae and is widely distributed throughout the tropical and subtropical regions, particularly Southeast Asia and India. Different parts of the plant, including leaves, fruits and bark, are traditionally used in folk medicine because of their various pharmacological properties. The leaves contain various phytochemical constituents such as flavonoids, alkaloids, phenolic compounds, tannins, terpenoids, coumarin etc. This exhibits antioxidant, anti-inflammatory and antimicrobial actions. The nervous system is particularly prone to oxidative stress due to high oxygen usage, high lipid levels and low levels of antioxidants. Excessive production of reactive oxygen species (ROS), including superoxide radicals, hydrogen peroxide and hydroxyl radicals, can cause oxidative stress, mitochondrial dysfunction, lipid peroxidation and neuronal cell death, responsible to the

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formation of neurodegenerative disorders such as Alzheimer's and Parkinson's diseases. H_2O_2 commonly used as an oxidative stress inducing agent in experimental models to determine cellular damage. The important constituents present in the *A.bilimbi* leaves may contribute neuroprotective effect by neutralizing free radicals, strengthening the body's natural antioxidant defence, preserving mitochondrial function, and preventing oxidative damage to cells. The neuroprotective potential of the leaf extract can be investigated using the MTT assay in SH-SY5Y Neuroblastoma cell lines exposed to H_2O_2-induced oxidative stress. Therefore, the present study aims to explore the neuroprotective potential of ethanolic *A. bilimbi* leaf extract prepared by the maceration method and evaluate its ability to protect neuronal cells against oxidative stress.

MATERIALS AND METHODS

3.1 Collection and Authentication

The leaves were collected from Kollam district, Kerala and Authenticated by taxonomist in SNCH (Sree Narayana College of Herbarium) and the voucher specimen (Acc No: 5186) was deposited in SNCH, Kerala, India. The leaves were washed with distilled water thoroughly to remove dirt particles and shade-dried at ambient temperature until a constant weight is obtained. Then the leaves are pulverized into coarse powder and stored in an air tight container.

3.2 Preparation of aqueous leaf extract

The dried leaves (40g) are macerated in ethanol (400ml) for 72 hours with occasional shaking to increase the efficiency of extraction. After maceration the mixture is filter through Whatman no. 1 filter paper. The filtrate is evaporated until a dry semi-solid extract formed.

3.3 Preliminary Phytochemical analysis

In the ethanol extract phytochemical screening is done and it contains phenols, flavonoids, tannins, alkaloids and terpenoids.

3.4 In vitro Neuroprotective activity

3.4.1 Principle

The MTT assay is a colorimetric technique. It is used to evaluate Cytotoxicity effects and cell viability. The conversion of yellow tetrazolium MTT into purple formazan crystals by metabolically active cells. This conversion reflects mitochondrial activity. It helps to determine the number of viable cells. The formed formazan crystals are dissolved using dimethyl sulfoxide (DMSO), and by using micro plate reader, the absorbance is measured.

3.4.2 Cell line and culture conditions

SH-SY5Y (Neuroblastoma) cells were purchased from the National Centre for Cell Sciences (NCCS), Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine, sodium bicarbonate and antibiotic solution. The cells were cultured in 25 cm^2 tissue culture flasks at 37°C in a humidified incubator with 5% CO_2.

3.4.3 Cell seeding and induction of oxidative stress

Following trypsinisation, SH-SY5Y cells were resuspended in 10% growth media. A 100µL volume ($5x10^3$ cells per well) of the cell suspension was placed within a 96 well plate and cells allowed to grow under standard conditions (37°C and 5% CO_2). Once cells had grown, 150µM H_2O_2 was introduced to the wells to simulate an oxidative stress. Cells were then incubated for 1 hour. Triplicate wells received freshly made extract at concentrations of 100, 50, 25, 12.5 and 6.25µg/mL. The diluent used was DMEM. Control wells were also included which contained untreated and H_2O_2 treated cells.

3.4.4 Preparation of Compound Stock

The sample was prepared into a stock solution by completely dissolving 1mg within 1ml of 0.1% DMSO and homogenising with a cyclomixer. The solution was then sterilised using a 0.22µm Millipore syringe filter. Cells were exposed to H_2O_2 and then treated with a range of concentrations of fresh extract (6.25, 12.5, 25, 50 and 100 µg/ml). The extract was diluted within DMEM. Each concentration was analysed in triplicate. Normal and H_2O_2 exposed cells were used as normal

and toxic controls. Cells were incubated within a humidified incubator at 37°C with 5% carbon dioxide for 24 hours.

3.5 Cytotoxicity Evaluation

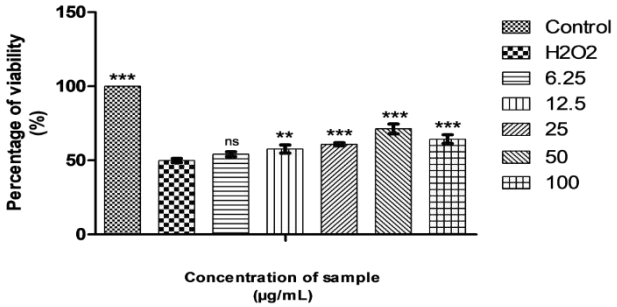
Cells were analysed following 24 hours of treatment. Morphological changes were used to assess cell damage. These included cell rounding, reduction in size, granulation and formation of vacuoles within the cytoplasm.

3.5.1 MTT cell viability assay

Following a 24-hour incubation period, the contents of the wells were removed, and 30 µL of reconstituted MTT solution was added to all test and control wells. The plate was gently shaken and incubated at 37°C in a humidified 5% CO_2 incubator for three hours. The supernatant was then removed and 100µl of DMSO was added to dissolve the formazan precipitate. Absorbance values were measured using a micro plate reader at a wavelength of 540 nm.

3.6 Evaluation of neuroprotective activity

The viability of the cell culture was assessed by comparing the mean OD values for both the extracts and the control. Increased cell viability in the extract treated cells compared to those treated with H_2O_2 was thought to suggest that the extracts have effective neuroprotective properties. .



RESULT AND DISCUSSION

4.1 EXTRACTION

The powdered *Averrhoa.bilimbi* (*A bilimbi*) leaf extract was obtained by maceration using ethanol as the solvent. The extract was concentrated to semisolid mass.

Parameter	observation
Weight of dried leaf powder	40g
Solvent used	95% ethanol
Extraction method	Maceration
Temperature	Room temperature
Duration	72 hours
Extract obtained	4.8

Parameter	observation
Percentage yield	12%

Extraction details

$$percentage\ yeild = \frac{\text{weight of extract}}{\text{weight of sample}} \times 100$$

Good extraction efficiency is obtained at 12% yield .The polar solvent ethanol effectively extracts phenolic compounds, flavonoids, terpenoids and alkaloids present *in A.bilimbi* leaves. Higher extract yield suggests a higher concentration of bioactive compounds responsible for antioxidant and neuroprotective activity.

4.2 PHYTOCHEMICAL SCREENING

Sl No:	Phytochemical	Test name	Observation	Result
1	Alkaloids	Dragendroff's test	Orange/Reddish brown precipitate	+
2	Flavonoids	Shinoda test	Pink/Red/Orange color	+
		Apigenin test	Intense yellow color	+
3	Phenols	Ferric chloride test	Blue/Green color	+
		Lead acetate test	White/Yellowish precipitate	+
4	Tannins	Ferric chloride test	Blue-Black color	+

Sl No:	Phytochemical	Test name	Observation	Result
5	Saponins	Foam test	Persistent froth	+
6	Terpenoids	Salkowski test	Reddish-brown color at interface	+
7	Coumarin	Alkaline test	Yellow color	+

A preliminary phytochemical screening was conducted on *A bilimbi* leaves to identify the primary classes of secondary metabolites present in the leaf. The result of phytochemical screening clearly shows that the leaf consists of various bioactive compounds. The presence of flavonoids, alkaloids, phenolic, tannins, terpenoids and coumarin strongly suggest that the sample has strong antioxidants, anti-inflammatory, antimicrobial and neuroprotective activity.

4.3 MTT Assay

MTT Assay was done for the evaluation. The Percentage of cell viability increased with increasing concentrations of the extract, showing a dose-dependent effect.

Cellline-SHSY5Y									
	OD1	OD2	OD3	Percentage viability1	Percentage viability2	Percentage viability3	Average	Stdev	Std error
Control	**0.4424**	**0.4517**	**0.4397**	**100**	**100**	**100**	**100**	**0**	**0**
H_2O_2	**0.2141**	**0.2308**	**0.2207**	**48.3951**	**51.0958**	**50.1933**	**49.8948**	**1.3749**	**0.7938**
Sample code-sample									
6.25	**0.2409**	**0.2513**	**0.2306**	**54.4529**	**55.6342**	**52.4448**	**54.1774**	**1.6124**	**0.9309**
12.5	**0.2689**	**0.2574**	**0.2435**	**60.7820**	**56.9847**	**55.3786**	**57.7152**	**2.7747**	**1.6020**
25	**0.2721**	**0.2694**	**0.2702**	**61.5054**	**59.6413**	**61.4509**	**60.8659**	**1.0608**	**0.6124**
50	**0.3051**	**0.3`52**	**0.3301**	**68.9647**	**69.7808**	**75.0739**	**71.2732**	**3.3167**	**1.9149**
100	**0.2853**	**0.2771**	**0.2958**	**64.4891**	**61.3460**	**67.2731**	**64.3694**	**2.9653**	**1.7120**

The study demonstrates that H_2O_2 significantly reduced the viability of SH-SY5Y neuronal cells indicating oxidative stress-induced cellular damage. Treatment with *Averrhoa bilimbi* leaf dependent increase from 6.25 to 50 µg/mL, with the maximum cell viability of 71.27% observed extract improved cell viability at all tested concentrations showing a gradual concentration- at 50 µg/mL. These finding suggests that *Averrhoa bilimbi* leaf extract exhibits potential neuroprotective activity against H_2O_2-induced oxidative stress in SH-SY5Y cells, with the highest protective effect is observed at 50 µg/mL.

CONCLUSION

Maceration produced an extract which showed good in vitro Neuroprotective effects in SH-SY5Y Neuroblastoma cells when exposed to hydrogen peroxide (H_2O_2) and subjected to analysis via the MTT assay. The highest level of protection was achieved at 50ug/mL (71.27%) proving that the maceration extract has good neuroprotective potential, and can provide protection for cells from oxidative stress. Overall the results indicate that *Averrhoa bilimbi* leaf extract is a potentially valuable natural product for use in the protection of neurons from oxidative stress.

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