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Original Article

Neuroprotective Effect of the Isolated Fraction from *Biophytum sensitivum* on Glutamate-Induced Neurotoxicity in HT22 Neuronal Cells

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ABSTRACT

Background: The present research paper elucidates to isolate specific fractions from *Biophytum sensitivum* and assess its ability to protect HT22 neuronal cells (immortalized mouse hippocampal neuronal cells) from glutamate induced neurotoxicity. **Materials and Methods:** Extraction method was employed for isolation of bioactive compounds from *Biophytum sensitivum*. These isolated fractions were administered to HT22 neuronal cells that had been subjected to glutamate-induced neurotoxicity. The neuroprotective effects isolated fractions were evaluated using a variety of assays and techniques, including cell viability assays, oxidative stress markers and morphological assessments. **Results:** *Biophytum sensitivum* extract demonstrated significant neuroprotective effects against glutamate-induced toxicity in HT22 cells. This neuroprotection may be attributed to the antioxidant properties of isolated compounds, as they were found to mitigate oxidative stress markers that are induced by glutamate. **Conclusion:** The results of this study are explained in this paper are with clear illustrations containing well demonstrated results. The findings suggest that *Biophytum sensitivum* holds promise as a rich source of neuroprotective agents and affords a reference version for further exploration of the isolated fractions could contribute to the development of therapeutic interventions for neurodegenerative conditions associated with glutamate-induced neurotoxicity.

Keywords: *Biophytum sensitivum*, Neuroprotective, Glutamate, Oxidative stress, Hippocampal cells, Antioxidant.

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INTRODUCTION

The field of neuroprotection has garnered significant attention due to its potential to combat neurodegenerative disorders and enhance overall brain health.[1] Among the various natural sources investigated for their neuroprotective properties, *Biophytum sensitivum*, a medicinal plant with a rich history in traditional medicine, has emerged as a promising candidate.[2] Glutamate, the main excitatory neurotransmitter in the central nervous system, plays an essential role in neuronal communication. An excessive glutamate release can result in neurotoxicity-a phenomenon implicated in various neurodegenerative conditions. Therefore, the discovery of compounds that alleviate glutamate-induced damage is crucial for the development of therapeutic approaches.[3,4] The isolated fraction from *Biophytum sensitivum* extract holds particular interest as it has demonstrated to contain potential neuroprotective effects in previous studies. This research paper aims to delve into the specific mechanisms underlying its protective actions against glutamate-induced neurotoxicity in HT22 neuronal cells.[5] HT22 is an immortalized mouse hippocampal neuronal cell line that lacks the cholinergic and glutamate receptors found in mature hippocampal neurons *in vivo*. This limits its use as a model for mature hippocampal neurons in memory-related research

Biophytum sensitivum, commonly known as the little tree plant or sensitive plant, is a small herbaceous plant native to tropical regions of Asia and Africa. It belongs to the Oxalidaceae family and is renowned for its rapid leaf movements in response to touch or mechanical stimuli, a phenomenon called thigmonasty. The plant typically grows in moist, shaded environments and can reach up to 30 cm in height. It produces small, yellow flowers

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and compound leaves that resemble miniature palm fronds. Traditionally used in Ayurvedic and Siddha medicine, *Biophytum sensitivum* is valued for its anti-inflammatory, antioxidant and wound-healing properties.[6]

Outcomes of the study

By explaining the neuroprotective potential of this isolated fraction, the authors seek to contribute valuable insights to the development of novel interventions for neurodegenerative disorders. The results of this investigation could illuminate the way for further exploration of *Biophytum sensitivum* and its bioactive components as potential candidates for neuroprotective therapies, fostering advancements in the field of neuroscience and providing hope for improved treatments for neurodegenerative conditions.[7,8] This study enlightens the identification of potential therapeutic agents derived from *Biophytum sensitivum* for the development of treatments for neurodegenerative disorders associated with glutamate-induced neurotoxicity in coming generations. Researchers might explore the potential synergies of the isolated fraction with existing neuroprotective agents or therapies, aiming to enhance overall treatment outcomes.

MATERIALS AND METHODS

Plant material

Biophytum sensitivum was collected from Rampachowdavaram forest in East Godavari and approved by Dr. Prasanna Kumari of the Department of Botany, D.N.R College, Bhimavaram. The Department of Pharmacology, Shri Vishnu College of Pharmacy, Bhimavaram maintained a sample voucher.

Plant Extraction Preparation

The specimen was air-dried at room temperature. The roots were extracted to a coarse powder (500 g). Fatty components were removed by petroleum ether at 60-80°C for a week. The residue is extracted with Soxhlet method with 95% methanol to form a methanol extract. The extract was evaporated at 30°C under reduced pressure for brownish-yellow extracts of *Biophytum sensitivum*.[9]

Isolation of fractions from extract

Fractions were isolated from *Biophytum sensitivum* by gradient solvent system using column chromatography as depicted in below Table 1.

Screening of Phytochemicals present in different fractions

Fractions of *Biophytum sensitivum* extracts (MEBS) were used for identification of different phytochemicals by using various phytochemical tests.

Cell Culturing

HT22 neurons were utilized in this study. The cells are cultured in Dulbecco's Modified Eagle's Medium and 10: v/v Fetal Bovine Serum, Penicillin: 1%, sodium bicarbonate: 2 mg/mL and HEPES, known as 2-[4-(2-hydroxyethyl) piperazin-1-yl] ethanesulfonic acid: 15 mM and 5% CO_2 at 37°C.

Determination of cell viability

MTT test is employed to estimate cellular metabolic activity.[10] NADPH dependant cellular oxidase, in some condition, indicate the number of living cells present. These enzymes convert the MTT tetrazolium dye to a precipitate form of purple colour.

The cells were inoculated into 48-well plates at 1.7x104/well and incubated for 24 hr at optimal temperature and 5% CO_2. Cells were treated for 1 hr with different fractions of *Biophytum sensitivum* (10, 100 µg/mL) and standard drug Trolox (50 µM). After 1 hr, 2 milli molar glutamate was added and incubated for 24 hr. Then, 1 mg/mL MTT was added to each well and the optical density at 570 nm was measured using an ELISA (Enzyme-linked immunosorbent assay) reader.

Estimation of Reactive oxygen species levels

Neuronal cell death is caused by the production of Reactive Oxygen Species (ROS) by oxidative stress. In this study, ROS production is estimated using 2'-7'-Dichlorodihydrofluorescein Diacetate (DCF-DA). Three samples of *Biophytum Sensitivitum*, 2 mM glutamate and Trolox treated cells for 8 hr. 10 µM DCF-DA was added to the cells $ incubated for 30 min at 37°C. The cells are then washed with Phosphate buffered saline after incubation. The fluorescence was measured at 490 nm.

Quantification of Ca^{2+} levels

Glutamate, an excitatory neurotransmitter acting on the N-Methyl-D-Aspartate (NMDA) receptor and increases the release of intracellular calcium, excess calcium mainly causes cell death. Fura-2-acetoxymethyl ester, often abbreviated Fura-2 AM, is a trans membrane derivative of a metric calcium indicator used to measure calcium levels in neurons. Cells were treated with different concentrations of BS, Trolox and 2 mM glutamate fractions. Then it went under incubation for 20 min after addition of 2 µM Fura-AM. Later, the cells were cleaned using HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer saline. Fluorescence was now measured at 380 nm to measure Ca^{2+} levels.

Measuring the Mitochondrial Membrane Potential (MMP)

Dye Rhodamine123 was employed for quantifying MMP in cells. Cells are subjected to different strengths of BS, Trolox and 2 mM glutamate fractions. 10 µM Rh 123 was added to the mixture and underwent incubation for 30 min. Later, the cells were cleaned

using saline solution of phosphate-buffer. The fluorescence was measured at 525 nm.

Estimation of Glutathione levels

The cells were subjected to treatment with various test compounds' concentrations of for 1 hr and glutamate 2 milli molar glutamate was added and incubated for 24 hr. Later, the cells were cleaned using saline solution of phosphate-buffer and centrifuged at 10000 rpm, 30 min at 4°C. The Supernatant liquid was treated with glutathione reagent. Measurement of Optical density is done at 560 nm with a colorimeter.[11]

Statistical Analysis

The results were interpreted statistically with the Standard error of the mean, represented as Mean±SEM. Statistical data is obtained with One-way ANOVA and *post hoc* test. The probability values, $p<0.001$ were considered as significant.

RESULTS

After performing various phytochemical analyses, the fraction A was found to contain steroids, phenolic and flavonoid compounds and the fraction B was found to contain flavonoid and steroidal compounds. The data were tabulated in Table 2.

Neuroprotective effect of *Biophytum sensitivum* on glutamate-induced oxidative cytotoxicity

Cell viable condition was estimated with MTT assay. The current study found that cells treated with 2 mM glutamate had lower cell viability (42.17±5.11) compared to normal cells. MEBS Fraction A (100 µg/mL) significantly increased cell viability (75.2±1.5%) compared to glutamate. Trolox significantly improved cell viability thereby protecting the cells from glutamate-induced cell death, as demonstrated below (Figure 1 and Graph 1).

Biophytum sensitivum inhibited glutamate-induced Reactive oxygen species accumulation

The paper depicted that, 2 mM glutamate treated cells increased ROS production (182.07±0.11) when compared to normal. Whereas MEBS Fraction A (100 µg/mL) of $^{***}p<0.01$ (Glutamate 2 mM) shows significant result than normal, $^{\#\#\#}p<0.001$ (Fraction A 100 µg/mL) showed more significant result than glutamate treated cells. Reactive Oxygen Species (ROS) production is significantly reduced (85±2.58) when compared to glutamate. The following graph (Graph 2), represented that the fraction of *Biophytum sensitivum* essentially protected cells from glutamate-induced cell death.

Biophytum sensitivum inhibited glutamate-induced Ca^{2+} influx

In current study 2 mM glutamate treated cells increased Ca^{2+} production (170.2± 0.11) when compared to normal. Whereas MEBS Fraction A (100 µg/mL) significantly reduced the Ca^{2+} production (130.3±1.85) when compared to glutamate. This result indicated that the fraction of BS essentially protected cells from glutamate-induced cell death (Graph 3).

The Effect of *Biophytum sensitivum* (BS) Fraction A on Mitochondrial Membrane Potential (MMP)

The researchers stated that the 2 mM glutamate treated cells decreased the Mitochondrial Membrane Potential (MMP) (74.2±0.89) when compared to normal. Whereas MEBS Fraction A (100 µg/mL) shows an increase in Mitochondrial Membrane Potential (MMP) (122.2±0.85) when compared to glutamate. This result specified the fraction of BS significantly protected the cells against glutamate induced cell death (Graph 4).

Table 1: Gradient solvent system isolation.

Solvent	Ratio	Fraction
Hexane	100%	--
Ethyl acetate	100%	--
Hexane: ethyl acetate	95:5	A
Hexane: ethyl acetate	70:30	B
Hexane: ethyl acetate	50:50	C
Methanol	100%	D

Table 2: Phytochemicals present in different fractions of MEBS.

Sl. No.	Phytochemical analyses	A	B	C	D	Hexane	Ethyl acetate
1	Alkaloids	x	x	x	x	x	x
2	Glycosides	x	x	x	x	x	x
3	Tannins	x	x	x	x	x	x
4	Phenols	✓	x	x	x	x	x
5	Flavonoids	✓	✓	x	x	x	x
6	saponins	x	x	x	x	x	x
7	Steroids	✓	✓	x	x	x	x

Here, ✓ denotes present and X denotes absent.

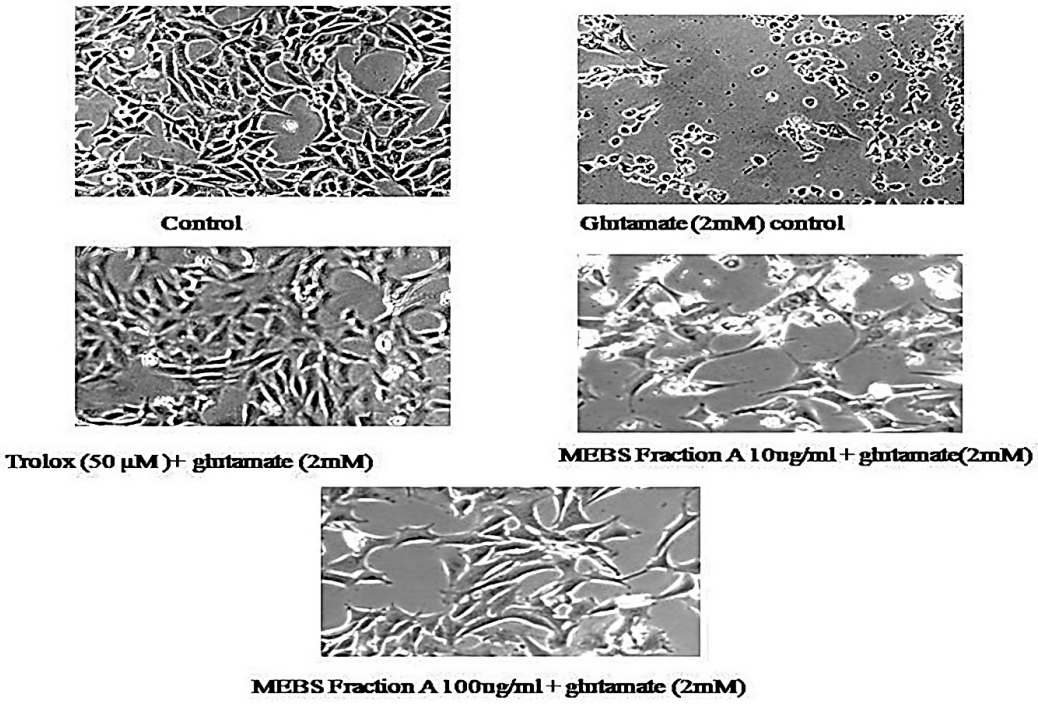


Figure 1: Neuroprotective effect of *Biophytum sensitivum* on glutamate-induced oxidative cytotoxicity.

Effect of *Biophytum sensitivum* (BS) Fraction on GSH

In this research, glutamate treated cells exhibited the reduced levels of GSH (30.18±3.18 %), when compared to normal. Whereas BS Fraction A (100 ug/mL) significantly increased the levels of GSH (62±0.58%) when compared to glutamate. This indicated the Fraction of BS significantly protected the neuronal cells from glutamate induced cell death (Graph 5).

DISCUSSION

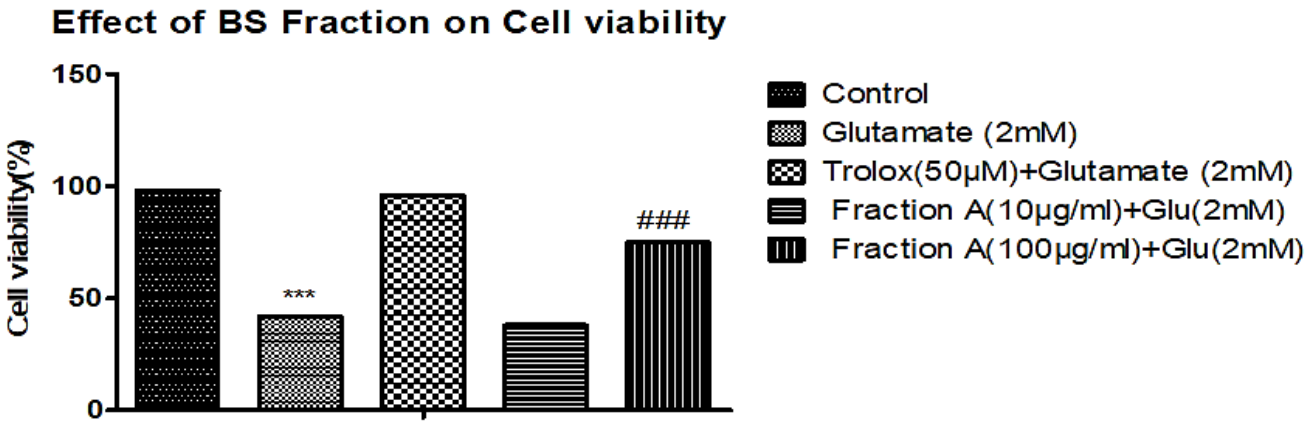
The glutamate is considered as vital neurotransmitter in CNS, playing a crucial role in various physiological processes such as learning and memory.[12] However, excessive release of glutamate and subsequent overstimulation of glutamate receptors can lead to excitotoxicity, a process implicated in various neurodegenerative disorders. The mechanism through which excessive glutamate can cause damage is by inducing oxidative stress.[13] This condition happens due to imbalance among ROS production and the capacity in detoxifying the same.

Glutathione (GSH), a crucial tri-peptide antioxidant makes key role in cell defence against oxidative stress. It consists of three amino acids: glutamate, cysteine and glycine. Glutathione is present in virtually all cells and is particularly abundant in the liver. Glutathione acts as an antioxidant by neutralizing Reactive Oxygen Species (ROS) and free radicals, thereby protecting cells from oxidative damage. It can directly scavenge free radicals or participate in enzymatic reactions that detoxify harmful compounds.[14] The importance of glutathione in cellular health and its multifaceted roles in antioxidant defence make it a critical molecule for overall well-being. Imbalances in glutathione levels have been associated with various health conditions, including neurodegenerative diseases, liver disorders and immune dysfunction.[15]

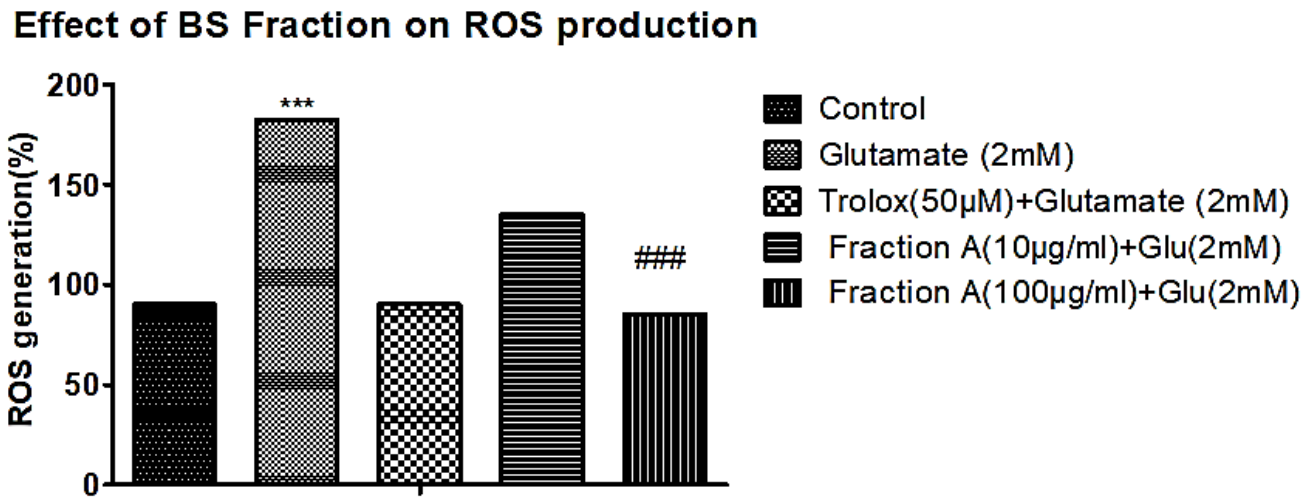
In this research, glutamate treated neuronal cells exhibited less existence of cells and elevated production of ROS, Calcium influx (Ca^{2+}) and disrupting mitochondrial membrane potential and decreased the levels of Glutathione. Whereas, the fractions of *Biophytum sensitivum* treated cells significantly enhanced the existence of cells, reduced ROS production, Ca^{2+} and normalise the mitochondrial membrane potential and increased the levels of GSH. This indicates fractions of *Biophytum sensitivum* protected the neuronal cells by oxidative damage induced by glutamate.

Our study envisages the fractions of *Biophytum sensitivum* have a significant neuroprotective action against glutamate induced cell injury due to its anti-oxidant effect. In future, *Biophytum sensitivum* could be valuable in various neuronal models and also helpful for drug discovery against neurodegenerative diseases.

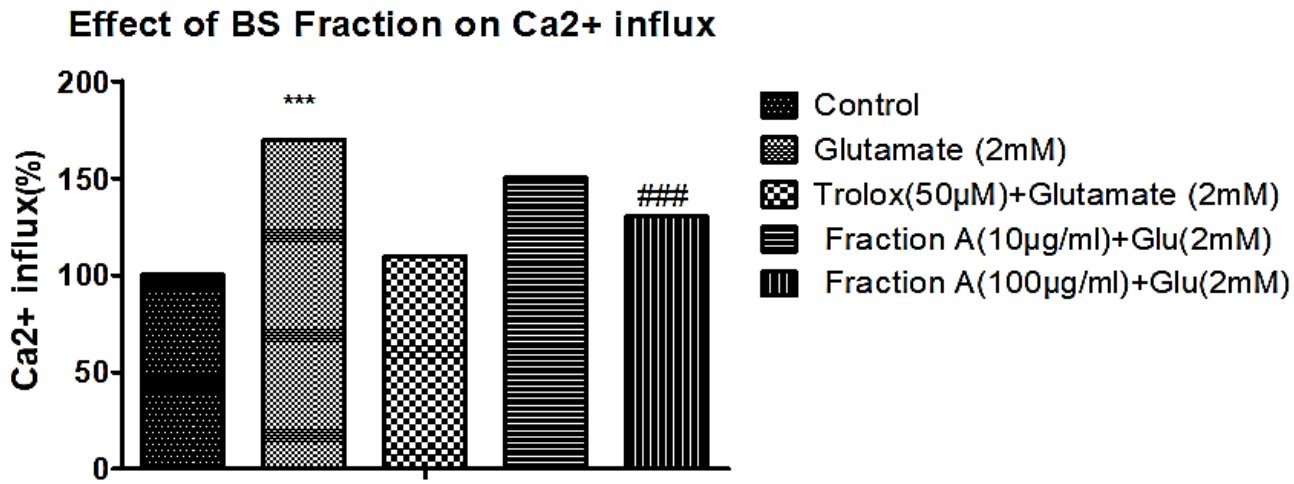
In this research, while a neuroprotective effect may be observed, the precise mechanisms underlying this effect may not be fully understood, limiting the ability to optimize the therapeutic potential. This study explores a neuroprotective effect; it could have significance for novel therapeutic agents' development to neurodegenerative diseases or conditions involving glutamate-induced neurotoxicity. Compounds derived from natural sources, such as *Biophytum sensitivum*, are often considered attractive due to their perceived safety and potential minimal side effects compared to synthetic drugs.



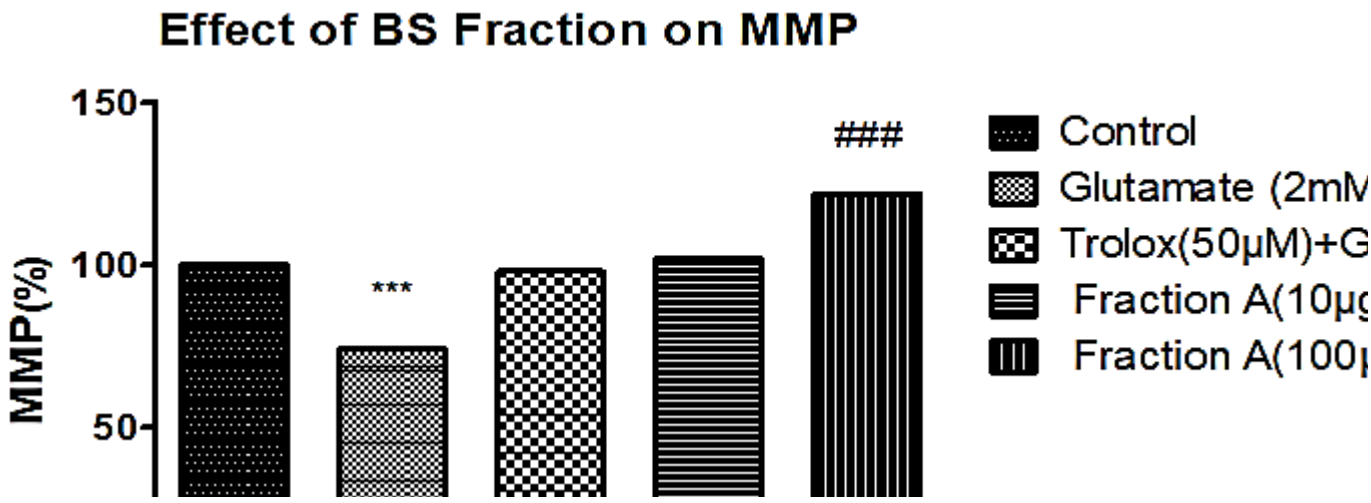
Graph 1: Effect of Fraction A of *Biophytum sensitivum* (BS) on cell viability. Glutamate has reduction in present relative cell viability than normal, whereas Trolox and *Biophytum sensitivum* (BS) showed an increase in % relative cell viability. The results are shown in triplicate. ###p<0.001(Fraction A 100 µg/mL) values are more significant than glutamate treated cells.



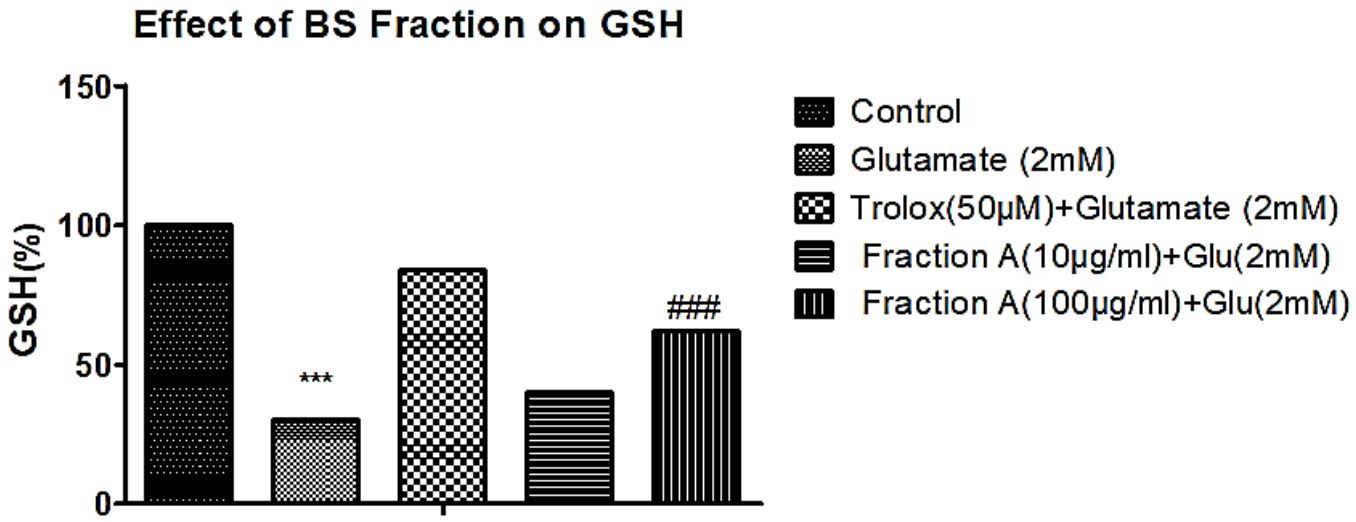
Graph 2: The influence of Fraction A of *Biophytum sensitivum* (BS) on Reactive Oxygen Species (ROS). Glutamate results in an increased percentage of ROS generation compared to normal levels, while Trolox and *Biophytum sensitivum* (BS) showed a little fall in the percentage of ROS generation. The values are presented in triplicate. Significance levels are denoted as ***p<0.01 (Glutamate 2 mM), indicating significance compared to normal and ###p<0.001 (Fraction A 100 µg/mL), emphasizing even greater significance compared to glutamate-treated cells.



Graph 3: The impact of Fraction A of *Biophytum sensitivum* (BS) on Ca^{2+} influx. Glutamate elevates the percentage of Ca^{2+} influx compared to normal levels, while Trolox and *Biophytum sensitivum* (BS) reduce it. The values represent the means of triplicate experiments. Significance levels are indicated as ***p<0.01 (Glutamate 2 mM), highlighting significance compared to normal and ###p<0.001 (Fraction A 100 µg/mL), underlining even greater significance compared to cells that are treated with glutamate.



Graph 4: The influence of Fraction A of *Biophytum sensitivum* (BS) on mitochondrial membrane potential. Glutamate diminishes mitochondrial membrane potential than normal, while Trolox and *Biophytum sensitivum* (BS) elevate it. Results are averaged from triplicate experiments. Significance levels are indicated as ***p<0.01 (Glutamate 2 mM), highlighting significance compared to normal and ###p<0.001 (Fraction A 100 µg/mL), emphasizing even greater significance compared to glutamate-treated cells.



Graph 5: Graph showing the impact of Fraction A of *Biophytum sensitivum* (BS) on Glutathione (GSH) levels. Glutamate reduced the GSH activity in comparison to normal levels, while Trolox and *Biophytum sensitivum* (BS) enhanced the GSH activity. Results are done in triplicate. The levels of significance are denoted as ***p<0.01 (Glutamate 2 mM), indicating significance compared to normal and ###p<0.001 (Fraction A 100 µg/mL), indicating even greater significance compared to glutamate-treated cells.

CONCLUSION

Based on the above results, it is implified that *Biophytum sensitivum* presents good neuroprotective action on HT22 cells that are subjected to oxidative stress mediated by antioxidant activity. Further studies are required to identify the pure compounds present in fractions and also to establish molecular mechanism for neuroprotective action.

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ABBREVIATIONS

MEBS: Methanolic extract of *Biophytum sensitivum*; **DMEM:** Dulbecco's modified eagle's medium; **FBS:** Fetal bovine serum; **HEPES:** N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; **MTT:** 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **ELISA:** Enzyme linked immune sorbent assay; **DCF-DA:** 2'-7'-Dichlorodihydrofluorescein diacetate; **NMDA:** N-methyl-D-aspartate; **MMP:** Mitochondrial membrane potential; **ROS:** Reactive oxygen species; **GSH:** Reduced glutathione.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

FUNDING/SUPPORT

No financial support received from government and non-government organisations.

ETHICAL CONSIDERATIONS

Not applicable as the cell lines were used in this study.

SUMMARY

Glutamate is a neurotransmitter, but excessive levels can be harmful and lead to cell death in neurons. This phenomenon is known as glutamate-induced neurotoxicity. HT22 cells are a type of mouse hippocampal neuron cell line commonly used in research to study neurotoxicity. In this work, isolated a specific fraction from *Biophytum sensitivum*, a plant traditionally used in medicine.

They exposed HT22 cells to glutamate to induce neurotoxicity and then treated them with the isolated fraction. The results showed that the fraction from *Biophytum sensitivum* protected the HT22 cells from glutamate-induced neurotoxicity. This study suggests that the isolated fraction from *Biophytum sensitivum* has potential neuroprotective properties and could be further investigated for its therapeutic potential in neurodegenerative diseases.

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