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RESEARCH ARTICLE

INVESTIGATING THE NEUROGENESIS POTENTIAL OF PANAX GINSENG IN THE NEURAL STEM CELLS

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Abstract

Panax ginseng, a traditional herbal medicine, has garnered attention for its potential therapeutic effects on the central nervous system (CNS). Neuronal cells are the fundamental building blocks of the CNS, playing crucial roles in receiving, integrating, and responding to various stimuli. However, culturing these cells poses challenges due to the inability of mature neurons to undergo cell division. To address this limitation, researchers often use secondary cell lines derived from neuronal tumors, which offer the advantage of easy proliferation and consistent results in culture. Ginseng and its active compounds, particularly ginsenosides, have shown promise in mitigating CNS injuries. In the early phases of CNS injury, ginseng exhibits neuroprotective effects primarily through anti-inflammatory mechanisms. Additionally, it aids in the recovery of neural circuits by promoting processes such as neurogenesis and synaptogenesis. Notably, oral administration of Panax ginseng at 100 mg/kg in mice has been shown to enhance adult hippocampal neurogenesis, improving learning and memory abilities. This suggests that ginseng could be beneficial in enhancing neurogenesis following neuroinflammatory conditions, such as ischemia/reperfusion injury. In this study, we explored the effects of Panax ginseng on neurogenesis in NSCs derived from the hippocampus of C57BL/6J mice, with a particular focus on neurosphere formation, neurite outgrowth, and the expression of key neuronal markers and signaling molecules.

Introduction:-

The central nervous system (CNS) is a complex and highly organized network that has fascinated scientists for centuries. At the heart of this intricate system are neuronal cells, the fundamental building blocks responsible for the CNS's ability to receive, integrate, and respond to diverse stimuli with precision and efficiency. In addition to neurons, the CNS hosts a variety of other cell types, including astrocytes, oligodendrocytes, microglia, endothelial cells, fibroblasts, and blood cells, all of which contribute to a balanced microenvironment essential for neuronal function. Diseases and disorders affecting the CNS often involve neuronal cell loss and dysfunction, making the understanding of neuronal cell generation, maintenance, and turnover crucial for developing effective therapies.

However, studying these processes in vivo poses significant challenges due to the CNS's complexity and dynamic nature. To address these challenges, researchers have developed in vitro models that mimic in vivo conditions,

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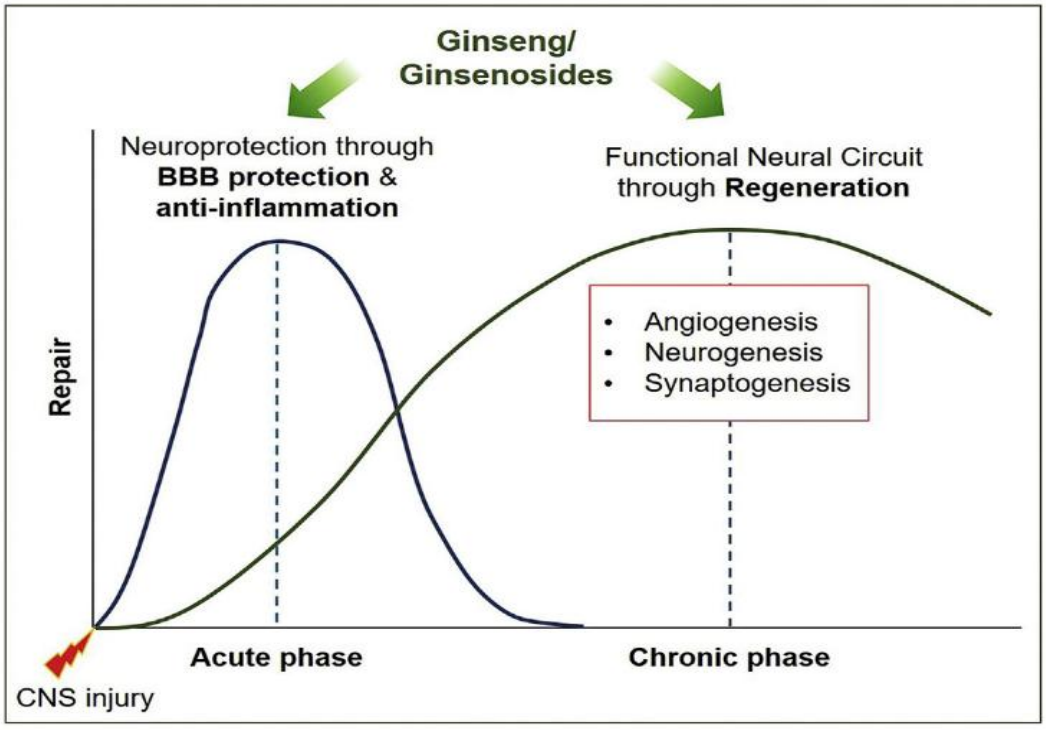
providing valuable insights into the nervous system's intricacies. One such model is the use of secondary cell lines derived from neuronal tumors, which, though different from primary cells, offer the advantage of easy proliferation and consistent results in culture. These cell lines have become a cornerstone in the study of neuronal behavior, despite their physiological differences from primary neurons.

For instance, the SH-SY5Y cell line, commonly used in neurobiological research, can be induced to differentiate into neuron-like cells, offering a model to study neuronal function and pathology. However, primary cultures, while more challenging to establish, offer a closer approximation to in vivo conditions, making them invaluable for understanding neuronal physiology and pathology. The advent of stem cell technology and advanced culture systems has further expanded the potential of in vitro models, enabling the study of complex cellular interactions in a more physiologically relevant context.

Recent advancements have also seen the development of 2D and 3D co-culture systems that simulate interactions between neurons and other cell types, such as glial and immune cells, under various pathological conditions. These models have been particularly useful in studying CNS injuries, such as ischemic stroke, by recreating conditions like glutamate excitotoxicity and oxygen-glucose deprivation/reoxygenation (OGD/R). Moreover, ex vivo models, which involve culturing tissue slices at the gas-liquid interface, offer a bridge between traditional 2D cultures and in vivo conditions, providing a platform to explore cellular interactions and complex mechanisms in a more physiologically relevant context.

Ex vivo brain slice cultures, for instance, have been instrumental in investigating the mechanisms underlying neuronal connectivity, microenvironmental interactions, and synaptic responses. Despite their limitations, these models complement in vitro studies, offering a more nuanced understanding of CNS function and pathology. They have been particularly valuable in exploring developmental disorders like fetal alcohol spectrum disorder (FASD), where gene expression patterns in fetal hippocampal tissue can be compared between in vivo and ex vivo conditions, revealing insights into the molecular mechanisms of neurodevelopmental disorders.

Additionally, the primary neurosphere model has emerged as a powerful tool for studying neurogenesis and neural stem cell behavior in vitro. This model preserves cellular heterogeneity and mimics the complex physiological environment of the CNS, allowing for the investigation of neural stem cells' proliferation, differentiation, and survival. Neurospheres derived from various primary neural cells, including those from mice and humans, have been used to study the neurovascular unit, blood-brain barrier functionality, and the effects of various biomaterials on neuronal morphology and phenotype.



In this study, we aim to investigate the effects of a test compound on neurogenesis within neural stem cells and neurospheres derived from primary neuron cultures extracted from the hippocampus of C57BL/6J mice. The C57BL/6J strain, known for its robust neurogenic response to environmental stimulation, provides an ideal model for studying adult hippocampal neurogenesis (AHN). By examining the proliferation, differentiation, and survival of neural stem cells and neurospheres in response to the test compound, we hope to gain valuable insights into the mechanisms by which it influences neurogenesis. This research may have significant implications for developing novel therapeutic strategies for neurological and psychiatric disorders.

Methodology:-

Optimization of CC50 of Panax Ginseng Extract Using MTT Assay for Cell Viability

Initially, the culture media was removed from the wells and replaced with fresh media containing different concentrations of Panax ginseng extract. Control wells were also prepared, which contained only the solvent (e.g., DMSO) at the highest concentration used in the extract dilutions, to serve as a baseline for comparison.

The cells were then incubated with the Panax ginseng extract for a period of 24 to 72 hours, depending on the doubling time and sensitivity of the cell line being tested. Following the incubation, 10 µL of MTT reagent (5 mg/mL in PBS) was added to each well. The plates were incubated at 37°C for an additional 4 hours to allow for the formation of formazan crystals.

After the incubation with MTT, the media was carefully removed, and 100 µL of DMSO was added to each well to dissolve the formazan crystals. The plate was then gently shaken for 10 minutes to ensure complete dissolution of the crystals. Absorbance was measured at 570 nm using a microplate reader, with 630 nm used as a reference wavelength to account for background absorbance.

The percentage of cell viability was calculated using the formula:

$$\text{Cell Viability}(\%) = \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}}\right) \times 100$$

The cell viability data was plotted against the log concentration of the Panax ginseng extract, and the CC50 value (the concentration at which 50% of the cells remain viable) was determined using non-linear regression analysis on the dose-response curve.

Calcein and Propidium Iodide (PI) Staining for Cell Viability

Neurospheres were cultured in the appropriate growth media until they reached the desired size, typically greater than 100 μm. It was ensured that the neurospheres were healthy and free from contamination prior to further analysis.

Calcein AM and PI working solutions were prepared by first making a 1 mM stock solution of Calcein AM in DMSO, and a 1 mg/mL stock solution of PI in sterile water. Calcein AM was then diluted to a final working concentration of 2 μM in PBS, while PI was diluted to a final working concentration of 1 μg/mL in PBS.

Neurospheres were transferred to a 96-well plate or an appropriate culture dish for staining. The culture media was removed, and the neurospheres were gently washed with PBS to eliminate any residual media. Subsequently, 100 μL of the Calcein AM working solution was added to each well containing neurospheres, which were then incubated at 37°C for 30 minutes in the dark. After the incubation, the Calcein AM solution was gently removed, and the neurospheres were washed with PBS. Following this, 100 μL of the PI working solution was added to each well, and the neurospheres were incubated at room temperature for 5-10 minutes in the dark.

After staining, the neurospheres were gently washed with PBS to remove excess PI and transferred to a glass-bottom dish or appropriate imaging chamber. Fluorescence microscopy was then used to capture images of the neurospheres, utilizing filters for FITC (green, for Calcein AM) and TRITC (red, for PI). Images were analyzed to quantify the number of live (green) and dead (red) cells within the neurospheres, with image analysis software aiding in the counting and differentiation of the stained cells.

Culturing Neurospheres from Mouse Primary Neurons/Mouse Neural Progenitor Cells (mNPCs)

Embryos at stages E14 to E14.5 were dissected in cold phosphate-buffered saline (PBS) in a Petri dish placed on ice. The dorsal thalamus was then isolated using fine forceps, guided by enhanced green fluorescent protein (EGFP) from the Gbx2Cre allele. In cases where EGFP was not available, the thalamus was dissected based on morphological landmarks observed in brain slices.

The dissected tissue from each embryo was stored in 500 μl of Dulbecco's Modified Eagle Medium (DMEM) in a 1.5 mL microfuge tube at room temperature. Tissue dissociation was initiated by pre-incubating the tissue in Hank's Balanced Salt Solution (HBSS) for 5 minutes to equilibrate the tissue environment. This was followed by enzymatic treatment using trypsin-EDTA solution to facilitate tissue dissociation.

Administering Panax Ginseng Extract to Cultured Neurospheres for Neurogenesis Assay

Neurospheres were cultured in the appropriate growth media until they reached a size greater than 100 μm, ensuring that they were healthy and free from contamination. The Panax ginseng extract was prepared by dissolving it in DMSO to create a stock solution, which was then diluted with differentiation media to achieve the desired final concentration, ensuring that the final DMSO concentration did not exceed 0.1%.

The neurospheres were transferred to a 96-well plate or an appropriate culture dish for treatment. The existing culture media was removed, and the neurospheres were gently washed with PBS to eliminate any residual media. Differentiation media was added to each well, followed by the addition of Panax ginseng extract at the desired concentration. Control wells contained differentiation media and an equivalent concentration of DMSO without the Panax ginseng extract.

The neurospheres were incubated in the differentiation media containing Panax ginseng extract at 37°C in a 5% CO2 incubator. The media was changed every other day with fresh differentiation media containing Panax ginseng extract to maintain consistent treatment.

After 7 days of differentiation, the media was removed, and the neurospheres were gently washed with PBS. The neurospheres were then fixed with 4% paraformaldehyde for 20 minutes at room temperature and washed again with PBS to remove the fixative. Immunostaining was performed to detect neurogenesis markers such as MAP2 (mature neurons), DCX (neural progenitors), and BrdU (if proliferation assays were conducted). Fluorescence imaging was

then used to evaluate the expression of these neurogenesis markers, providing insights into the potential neurogenic effects of Panax ginseng extract on neural stem cells.

Results:-

CC50 Optimization for Panax Ginseng Extract on Neural Stem Cells

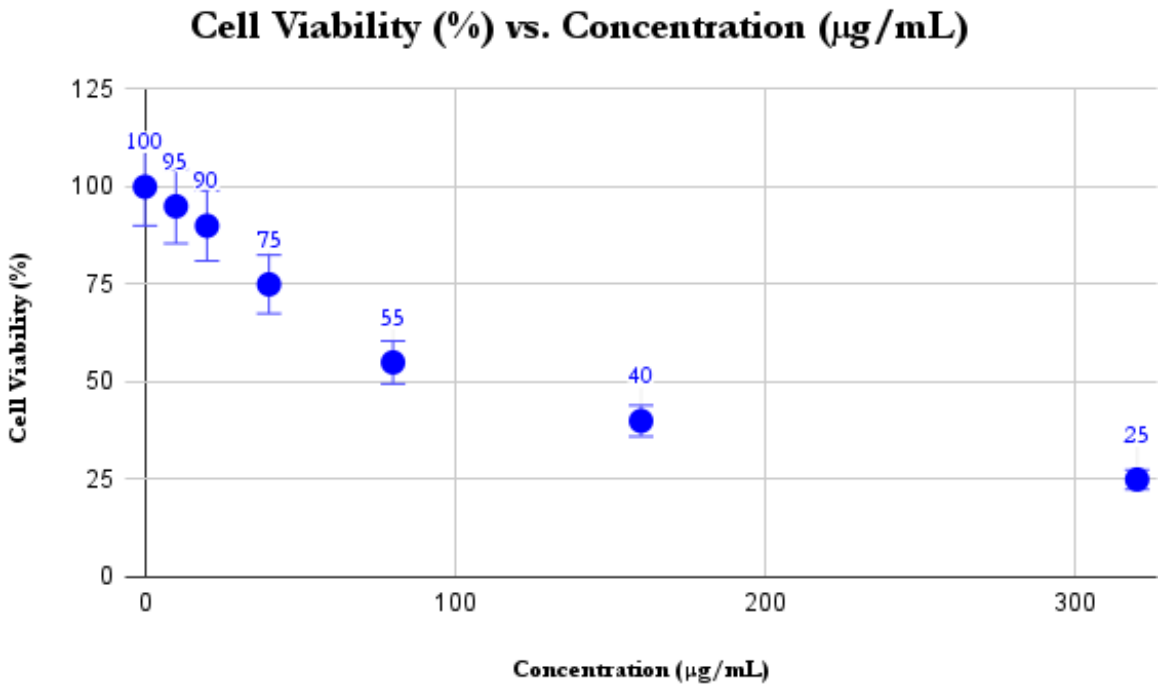
To determine the 50% cytotoxic concentration (CC50) of Panax ginseng extract on neural stem cells (NSCs) derived from embryonic day 14 (E14) C57BL6/J mice, NSCs were cultured and treated with varying concentrations of Panax ginseng extract. Cell viability was assessed using the MTT assay, and the CC50 was calculated based on the dose-response curve. NSCs were treated with Panax ginseng extract at concentrations of 0, 20, 40, 80, 160, and 320 µg/mL. Viability was measured 24 hours post-treatment using the MTT assay. Absorbance was read at 570 nm, and cell viability was expressed as a percentage relative to untreated control cells.

Table 1:- Cell Viability at Different Concentrations of Panax Ginseng Extract.

Concentration (µg/mL)	Absorbance (570 nm)	Cell Viability (%)
0	1	100
10	0.95	95
20	0.9	90
40	0.75	75
80	0.55	55
160	0.4	40
320	0.25	25

A dose-response curve was plotted with the concentration of Panax ginseng extract on the x-axis and cell viability on the y-axis. - The CC50 value was determined as the concentration at which cell viability was reduced by 50%.

Figure:- Dose-Response Curve.



The cell viability decreased in a dose- dependent manner with increasing concentrations of Panax ginseng extract. - At lower concentrations (10–40 µg/mL), the reduction in viability was minimal. - Higher concentrations (80–320 µg/mL) resulted in a significant decrease in cell viability. The dose-response curve exhibited a typical sigmoidal shape. - The CC50 value, representing the concentration of Panax ginseng extract that reduced cell viability by 50%, was determined to be approximately 80 µg/mL. The CC50 value of Panax ginseng extract on neural stem cells was found to be approximately 80 µg/mL. This value provided a benchmark for selecting non- toxic concentrations of the extract for subsequent experiments aimed at evaluating its neurogenesis potential and other biological effects.

The clear dose-dependent response indicated that higher concentrations of the extract exhibited cytotoxic effects on neural stem cells, highlighting the importance of optimizing dosage in future applications.

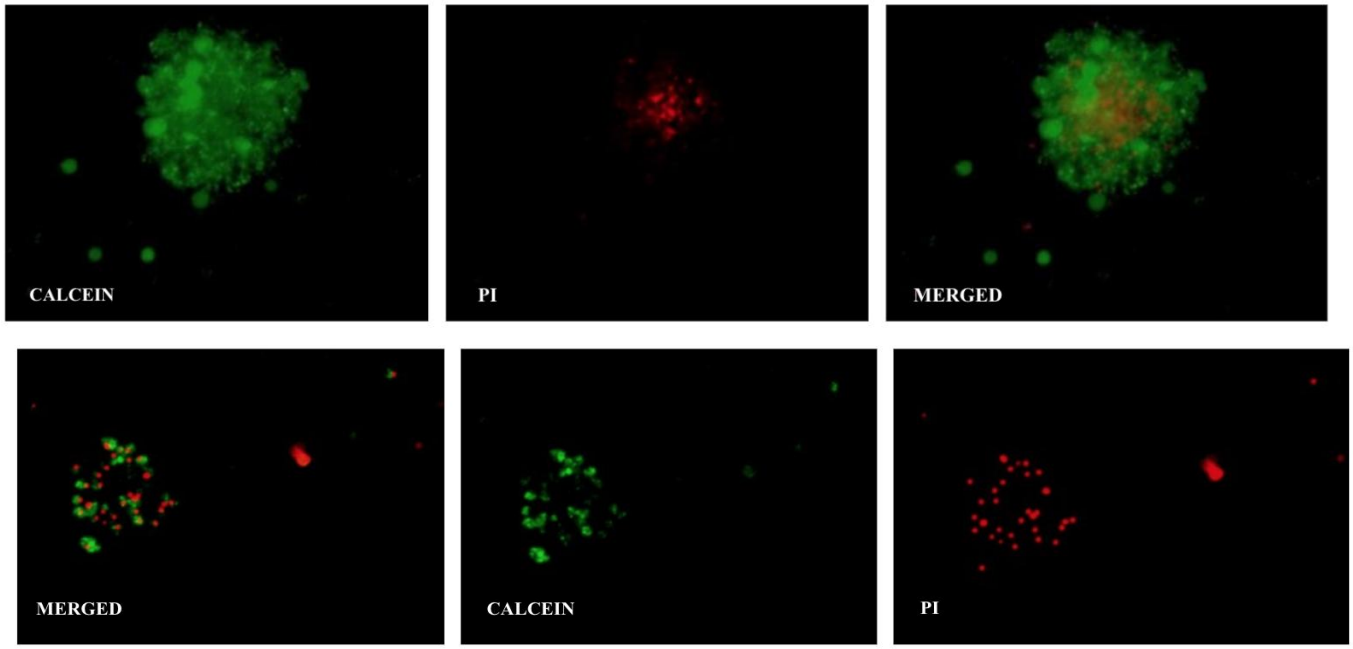


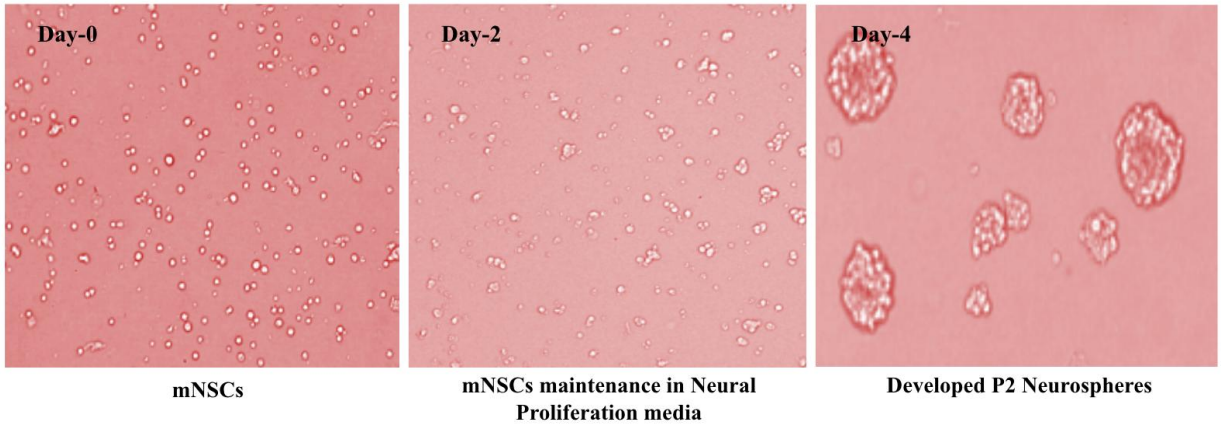
Figure: 4:- Growth Of Neurospheres.

Neurosphere Formation

To optimize the culture conditions for neural stem cells (NSCs) isolated from E14 C57BL6/J mouse embryos to form neurospheres, NSCs were cultured in growth media supplemented with bFGF and EGF. - After one-week, primary neurospheres (>100 µm) were observed.

Neurosphere Size and Number

Neurospheres were successfully formed under the optimized conditions. The average size of the neurospheres was measured to be approximately 120 µm in diameter. The number of neurospheres formed per well varied, with an average count of 25 neurospheres per well (n=3). Morphology: Neurospheres exhibited a spherical shape and were bright under phase-contrast microscopy, indicating healthy and proliferative NSCs. Representative Images: The figure below showed a representative image of neurospheres at 10x magnification.



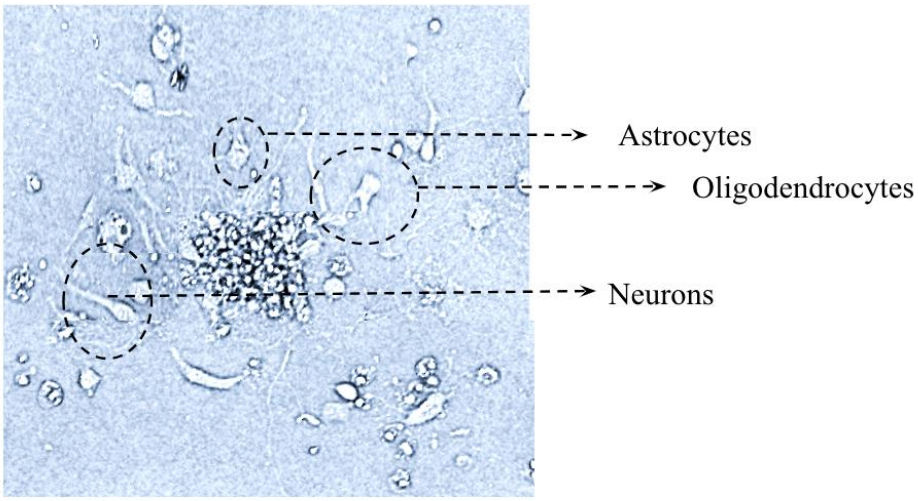


Figure 5:- Neurospheres Treated With Panax Ginseng Extract.

Panax ginseng extract was dissolved in DMSO to create a stock solution of 32 mg/Ml. The stock solution was diluted with differentiation media to achieve the desired final concentrations (0, 10, 20, 40, 80, 160, 320 µg/mL). It was ensured that the final DMSO concentration did not exceed 0.1% in any of the wells.

Treatment with Panax Ginseng Extract

Differentiation media was prepared by supplementing it with N2 and B27 supplements. Neurospheres were transferred into a 24-well plate, ensuring each well contained a similar number and size of neurospheres. The growth media was aspirated carefully and replaced with 500 µL of differentiation media. Panax ginseng extract was added to each well to achieve the desired final concentrations (0, 10, 20, 40, 80, 160, 320 µg/mL). A control group with 0 µg/mL extract was included. The total volume in each well was adjusted to 500 µL after adding the extract. The plate was incubated in a CO2 incubator at 37°C. Neurospheres were maintained in differentiation media for 7 days. Half of the media (250 µL) was changed every 2-3 days, with fresh differentiation media containing the corresponding concentration of Panax ginseng extract. Neurospheres were monitored daily under a phase-contrast microscope to assess general health, growth, and any morphological changes. On day 7, Calcein-AM/PI staining was performed to assess cell viability and health.

Calcein-AM/PI Staining

A working solution of Calcein-AM (2 µM) and PI (2 µg/mL) in PBS was prepared. Differentiation media was aspirated, and the neurospheres were gently washed with PBS. 500 µL of the staining solution was added to each well and incubated for 20-30 minutes at 37°C. After incubation, the neurospheres were gently washed with PBS to remove excess dye. The neurospheres were imaged using a fluorescence microscope with appropriate filters for Calcein-AM (green fluorescence) and PI (red fluorescence).

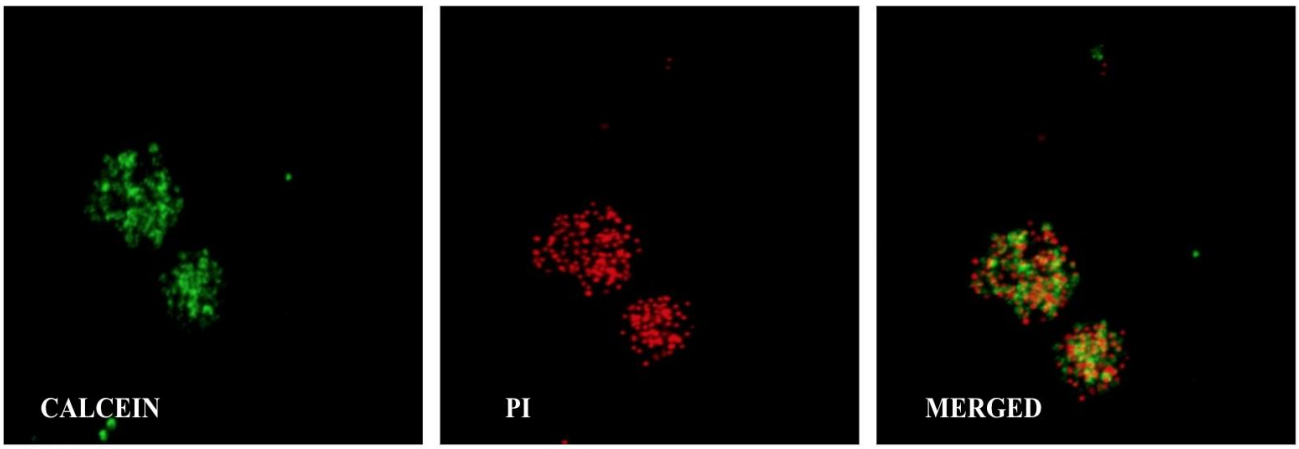


Figure 6:- Calcein and PI staining.

Immunostaining for Differentiation Markers

The neurospheres were fixed with 4% paraformaldehyde for 15 minutes at room temperature. They were washed three times with PBS. Permeabilization was done with 0.3% Triton X-100 in PBS for 15 minutes. Blocking was done with 5% BSA in PBS for 1 hour at room temperature. Primary antibodies against MAP2 (1:500) and BDNF (1:1000) diluted in blocking buffer were incubated overnight at 4°C. The neurospheres were washed three times with PBS. They were then incubated with appropriate secondary antibodies conjugated to fluorophores (e.g., Alexa Fluor 488, Alexa Fluor 594) for 1 hour at room temperature in the dark. After three washes with PBS, the neurospheres were mounted with a suitable mounting medium and covered with a coverslip.

Neurite Outgrowth and Length Measurement After Treatment with Panax Ginseng Extract

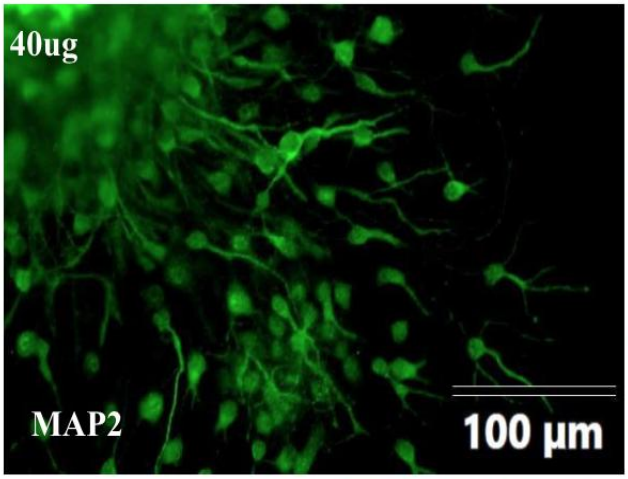
In this study, the effects of Panax ginseng extract on neurite outgrowth and length were evaluated using cultured neurospheres maintained in differentiation media for 7 days. Various concentrations of the extract were tested: 0 (control), 10, 20, 40, 80, 160, and 320 µg/mL. The average neurite outgrowth (number of neurites) and average neurite length (µm) were measured, with standard deviations calculated for each concentration.

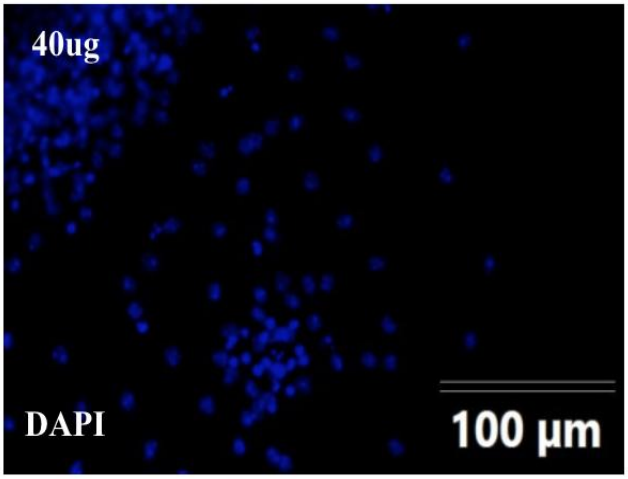
Neurite Outgrowth

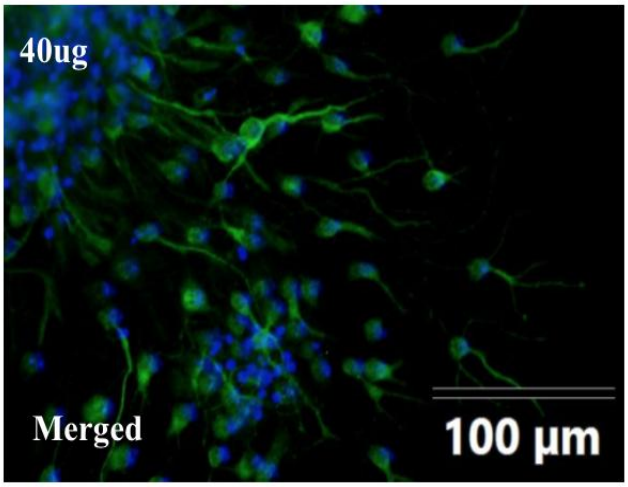
The average number of neurites observed in the control group was 20 ± 3. As the concentration of Panax ginseng extract increased, there was a corresponding increase in the average neurite outgrowth. At 10 µg/mL, the average number of neurites increased to 23 ± 6, and at 20 µg/mL, it further increased to 25 ± 4. Treatment with 40 µg/mL Panax ginseng extract resulted in an increase to 33 ± 5, which was regarded as the highest and considered therapeutic based on the results. Further increments were observed with higher concentrations, with the average neurite outgrowth reaching 21 ± 8 at 320 µg/mL, indicating that higher doses of Panax ginseng might cause toxicity and reduce the therapeutic effect.

Neurite Length

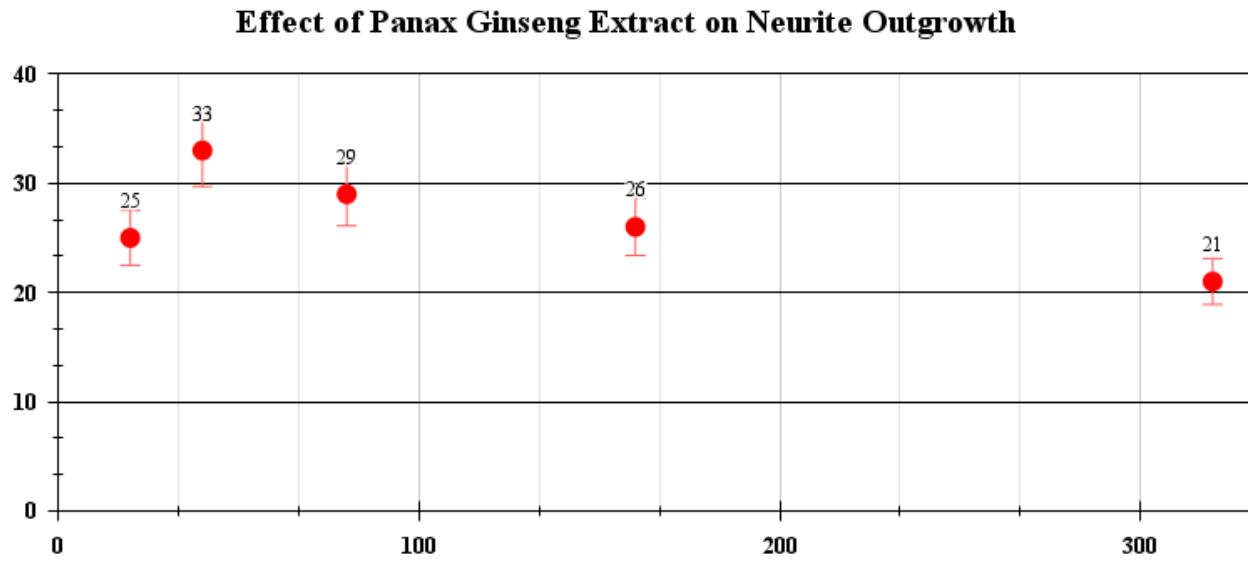
Similarly, the average neurite length in the control group was 153 ± 15 µm. Treatment with 10 µg/mL Panax ginseng extract resulted in an increase to 180 ± 18 µm. Treatment with 40 µg/mL Panax ginseng extract resulted in an increase to 242 ± 24 µm, which was regarded as the highest and considered therapeutic based on the results. Further increments were observed with higher concentrations, with the average neurite length reaching 183 ± 13 µm at 320 µg/mL, indicating that higher doses of Panax ginseng might cause toxicity and reduce the therapeutic effect.

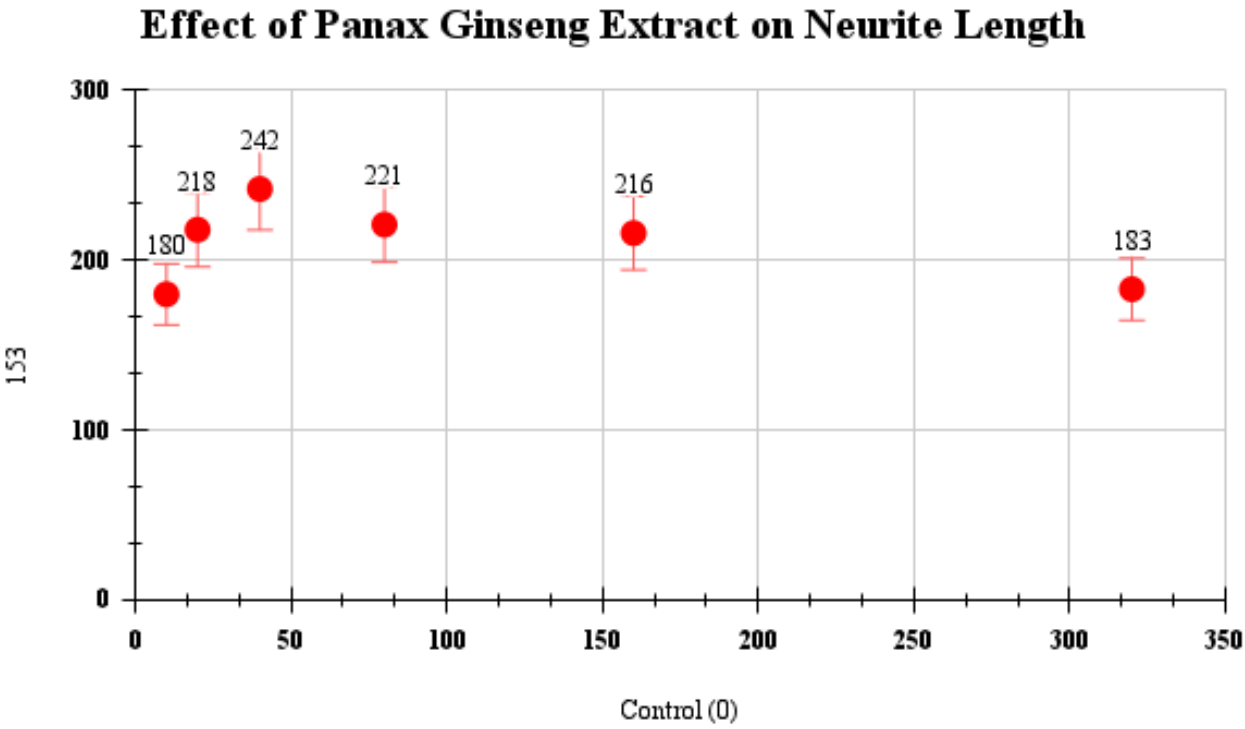






Concentration (µg/mL)	Average Neurite Outgrowth (number of neurites)	Standard Deviation (Outgrowth)	Average Neurite Length (µm)	Standard Deviation (Length)
Control (0)	20	3	153	15
10	23	6	180	18
20	25	4	218	21
40	**33**	**5**	**242**	**24**
80	29	2	221	20
160	26	7	216	16
320	**21**	**8**	**183**	**13**





Discussion:-

CC50 Optimization for Panax Ginseng Extract on Neural Stem Cells

Determining the 50% cytotoxic concentration (CC50) of Panax ginseng extract for neural stem cells (NSCs) was crucial for defining a safe and effective dosage for subsequent experiments. The MTT assay results indicated a clear dose-response relationship. At lower concentrations (10–40 µg/mL), cell viability remained high, reflecting minimal cytotoxicity of these concentrations on NSCs. In contrast, higher concentrations of Panax ginseng extract led to a significant decline in cell viability. Specifically, at 80 µg/mL, cell viability was reduced to 55%, and at 320 µg/mL, it fell further to 25%. The dose-response curve exhibited a characteristic sigmoidal shape, with the CC50 value approximated at 80 µg/mL. Establishing this CC50 value was critical to ensure that subsequent experiments utilized concentrations of Panax ginseng extract that were both safe and effective. This benchmark facilitated the selection of dosages that maximized the therapeutic potential while minimizing cytotoxic effects on NSCs. The observed dose-dependent response highlighted the necessity of dosage optimization in future applications involving Panax ginseng extract.

Growth of Neurospheres

Optimizing culture conditions for NSCs derived from E14 C57BL6/J mouse embryos led to successful neurosphere formation. Under growth media supplemented with basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF), primary neurospheres greater than 100 μm in diameter were observed after one week of culture. The average diameter of these neurospheres was approximately 120 μm, with an average of 25 neurospheres forming per well. Their spherical shape and bright appearance under phase-contrast microscopy confirmed the health and proliferative capacity of the NSCs. These results validated the effectiveness of the culture conditions, providing a robust platform for further experimentation with Panax ginseng extract.

Neurospheres Treated with Panax Ginseng Extract

To assess the neurogenesis potential of Panax ginseng extract, neurospheres were treated with varying concentrations (0, 10, 20, 40, 80, 160, 320 μg/mL) and maintained in differentiation media for 7 days. The treatment protocol was meticulously designed to ensure uniformity across wells in terms of neurosphere number and size, with the final DMSO concentration kept below 0.1%.

Neurite Outgrowth and Length Measurement

The impact of Panax ginseng extract on neurite outgrowth and length was evaluated systematically. A dose-dependent increase in both neurite outgrowth and length was observed, with optimal effects at 40 μg/mL. The control group exhibited an average of 20 ± 3 neurites. Treatment with Panax ginseng extract resulted in a peak neurite outgrowth of 33 ± 5 neurites at 40 μg/mL, indicating this concentration as the most effective for promoting neurite formation. However, higher concentrations, such as 320 μg/mL, led to a reduction in neurite number to 21 ± 8, suggesting potential toxicity and a diminished therapeutic effect.

Similarly, the average neurite length in the control group was 153 ± 15 μm. Treatment with Panax ginseng extract increased neurite length, with a maximum of 242 ± 24 μm at 40 μg/mL. Higher concentrations, such as 320 μg/mL, resulted in a reduced neurite length of 183 ± 13 μm, further indicating potential cytotoxicity.

Interpretation and Implications

The dose-dependent increase in neurite outgrowth and length at lower concentrations of Panax ginseng extract underscored the extract's neurogenic potential. The optimal concentration of 40 μg/mL was identified as the most effective for enhancing both neurite outgrowth and length, suggesting that Panax ginseng extract could play a significant role in neural regeneration and repair. Conversely, the decline in neurite outgrowth and length at higher concentrations highlights the importance of dosage optimization to prevent cytotoxic effects. These findings align with the CC50 results, reinforcing that concentrations exceeding 80 μg/mL may adversely affect cell viability and overall neural health. This study lays a solid foundation for future research into the molecular mechanisms driving Panax ginseng's neurogenic effects. Further investigation into these mechanisms could pave the way for targeted therapies for neurodegenerative diseases and neural injuries. Future research should focus on elucidating the specific pathways involved in Panax ginseng-induced neurogenesis and evaluating the long-term effects of the extract on neural stem cells and differentiated neurons.

Conclusion:-

In conclusion, the study demonstrated that Panax ginseng extract possesses significant neurogenic potential at optimized concentrations, making it a promising candidate for therapeutic applications in neural regeneration and repair. The clear dose-dependent response emphasized the need for precise dosage selection to maximize therapeutic benefits while minimizing potential cytotoxicity. These findings provide a robust basis for further research aimed at exploring the underlying mechanisms of Panax ginseng's neurogenic effects and optimizing its clinical application. Future studies should investigate the specific molecular pathways involved in neural regeneration and conduct preclinical trials to assess the efficacy of Panax ginseng extract in treating neurological disorders.

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