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NEUROPROTECTIVE POTENTIAL OF GC–MS CHARACTERIZED PHYTOCONSTITUENTS FROM ALSTONIA VENENATA LEAVES: INTEGRATED IN-SILICO AND IN-VITRO EVALUATION TARGETING THE PI3K/AKT SIGNALLING PATHWAY

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Abstract:
Alzheimer's disease (AD) is a neurodegenerative disorder that advances gradually over a long period, where amyloid-beta aggregates cause oxidative stress-related disease conditions, neuroinflammation, dysfunction of mitochondria, and neuronal apoptosis. Current pharmacotherapies provide merely symptomatic relief and have been implemented with blood-brain barrier inhibitory effects as well as adverse side effects. Therefore, a novel need for multi-target neuroprotective natural agents is increasing. This paper sought to assess the neuroprotective capacity of Alstonia venenata leaf extract following an overall in-silico, in-vitro, and mechanistic methodology. The analysis of the ethanol extract was done using GC-MS to profile the phytochemicals. Swiss ADME was used to assess drug-likeness and blood-brain barrier permeability. The analyses included target prediction, network pharmacology, protein-protein interaction, Gene ontology and KEGG pathway. Molecular docking was done towards Akt1, GSK-3β, and β-amyloid. The neurotoxicity induced by SH-SY5Y cells by AlCl3 was evaluated by using cell viability through MTT and caspase-3 ELISA measures. Nine out of 27 identified phytoconstituents met the BBB permeability and Lipinski criteria. The network analysis found 355 overlapping AD targets with hub genes being AKT1, CASP3, GSK3B, and MAPK1. KEGG analysis also revealed the PI3K-Akt pathway to be the most prominent neuroprotective mechanism. Molecular docking demonstrated that β-carotene, sitosterol, squalene, and quinic acid have high affinities with Akt1 and GSK-3β. In-vitro response demonstrated that the cell viability increased with a dose up to 400 µg/mL, which provided the protection of nearly 95% and had a significant effect on caspase-3 activity. Alstonia venetata presents strong neuroprotective activity, which activates PI3K-Akt-GSK-3b-caspase signalling pathway, which justifies its application as a valuable anti-Alzheimer therapeutic target. The major bioactive compounds identified included β-sitosterol ($C_{29}H_{50}O$), squalene ($C_{30}H_{50}$), quinic acid ($C_7H_{12}O_6$), α-linolenic acid ($C_{18}H_{30}O_2$), and β-carotene ($C_{40}H_{56}$), which were further evaluated for drug-likeness, molecular docking, and biological activity.

Keywords: Alstonia venenata; Neuroprotection; Alzheimer's disease; PI3K/Akt pathway; SH-SY5Y cells

1. Introduction

1.1 Neurodegenerative Disorders Overview

Alzheimer's, Parkinson's, and Huntington's neurodegenerative disorders, among others, are caused by loss of certain populations of neurons with time and result in cognitive and motor dysfunction. The main findings are the existence of abnormal protein aggregates, such as amyloid plaques in Alzheimer's disease and α-synuclein in Parkinson's disease, that impair the functioning of neurons and propagate along the neural pathways [1,2]. Neuronal damage and synaptic death are also caused by oxidative stress and depletion of neurotransmitters (especially dopamine depletion in Parkinson's), in particular [3,4]. An important role in the slow loss of neurons that occurs in these diseases is played by the process of apoptosis or programmed cell death [4]. Activated microglial and astrocyte release of pro-inflammatory cytokines, as a factor that increases neuronal damage, is common to these disorders and worsened by neuroinflammation [2,5]. Although there is more knowledge about these mechanisms, the existing treatment strategies focus on symptoms, and there are no curable options available, which is why innovative treatment methods that would target these underlying pathological processes are required [3,4].

1.2 Limitations of Current Therapies

Existing treatments of neurodegenerative diseases, such as Alzheimer's and Parkinson's, such as donepezil, rivastigmine and memantine, are mainly aimed at symptomatic rather than disease-modifying effects [4,6,7]. One of the significant constraints is the blood-brain barrier (BBB), which filters out almost 99 per cent of medications to the brain, severely limiting the ability of many drugs to improve therapy [4,6]. Moreover, the toxicity and other adverse effects of these drugs may also occur as a result of their long-term use, which makes treatment even harder [4,8]. The majority of accepted treatments are aimed not at the underlying pathogenesis but at the modulation of neurotransmitters or the management of the symptoms [9]. Newer approaches like nanotherapeutics and more efficient types of delivery like intranasal administration will address the challenge of the BBB, but remain in the research phase [6,7]. On balance, the intricacy of neurodegenerative diseases and obstacles to delivering the drugs to the brain explain an emergency in finding novel remedies, on top of existing symptomatic ones [4,10].

1.3 Role of Medicinal Plants in Neuroprotection

Medicinal plants have high neuroprotective properties because of their polypharmacological nature, or their ability to act on various pathological mechanisms at the same time, including oxidative stress, apoptosis, and inflammation [11,12]. They contain bioactive substances that exert effects on neuronal status and neurogenesis: antioxidant, anti-apoptotic, and anti-inflammatory effects of their bioactive compounds that include polyphenols and flavonoids, as well as, terpenoids [13-15]. The natural compounds have the ability to penetrate the blood-brain barrier and regulate the signal transduction to improve neuronal survival, decrease protein aggregation, and inhibit neuroinflammation [13,15,16]. There is some evidence suggesting that traditional medicinal herbs can help enhance memory, motor performance and dissipate neurodegenerative damage in experimental models [17-19]. Moreover, extracellular vesicles of plant origin offer a new method for delivery, which is effective in improving bioavailability and therapeutic effect with low toxicity [16]. Generally, phytotherapy is a promising complementary treatment in managing neurodegenerative diseases because the therapy tackles the various disease pathologies with fewer adverse effects compared to traditional medications [11,12].

1.4 Alstonia venenata – Ethnomedicinal & Pharmacological Background

Alstonia venenata is in the Apocynaceae family, traditionally used across many tribal communities and also in Ayurveda to treat central nervous system (CNS) conditions, including epilepsy, insanity, and fever [20,21]. Several bioactive compounds in the plant include 2-carbolines, coumarin, tannins, flavonoids and phenols [20,22]. In ethnomedicine, the stem bark, root bark, leaf and fruit are used to treat both neurological disorders and poisons [20,23]. It has been shown to have anticonvulsant and antidepressant effects and experimentally, it has been found to be effective in preventing seizures and protecting the brain [21]. Alstonia venenata has also been shown to have cytotoxic activity against cancer infections and antioxidant properties, and this means it can be used in a wider range of therapeutic treatments [24]. These results outline its complex pharmacological history based on traditional practice and contemporary scientific confirmation [20,21,24].

Previous phytochemical investigations of Alstonia venenata have reported the presence of several bioactive constituents including terpenoids, sterols, flavonoids and fatty acids. However, the neuroprotective potential of individual phytoconstituents such as β-sitosterol, squalene, quinic acid and α-linolenic acid has not been systematically explored in Alzheimer's disease models. Identification and characterization of these compounds is essential to establish reproducible pharmacological activity.

1.5 Rationale and Objective of the Study

Irrespective of substantial studies of plant-based neuroprotective agents, Alstonia venenata has yet to be scientifically demonstrated by an integrated in-silico, in-vitro, and mechanistic method of neurodegenerative disorders. Attaining phytochemical targets to molecular signalling and neuronal survival is the correlation that is lacking in studies, which is a significant research gap.

The objectives are:

1.To evaluate the neuroprotective potential of Alstonia venenata leaf extract through integrated in-silico and in-vitro approaches.

2.To identify and screen bioactive phytoconstituents using GC–MS analysis and drug-likeness prediction.

3.To predict potential Alzheimer's disease-related molecular targets using bioinformatics tools.

4.To elucidate the involvement of the PI3K/Akt signalling pathway through network pharmacology and molecular docking studies.

5.To assess the cytoprotective effect of the extract against $AlCl_3$-induced neurotoxicity using the MTT assay.

6.To evaluate the anti-apoptotic potential of the extract by measuring caspase-3 activity in SH-SY5Y cells.

1.6 Research Gap

Although there is ethnomedicinal relevance between phytochemical profiling and network pharmacology of the Alstonia venenata, no study has combined the effects of analysis of the phytochemical with the molecular docking and in vitro validation to explain the mechanistic role of the plant in Alzheimer disease related neurodegeneration. This paper will fill this gap by reviewing its multi-target neuroprotective capabilities systematically, and paying special focus to the PI3K/Akt signalling pathway.

2. Materials And Methods

2.1 Plant Collection And Authentication

The fresh leaves of Alstonia venenata were taken in the area of Vandiperiyar, Idukki District, Kerala, India. Authentication of the plant material was carried out by a qualified taxonomist, and a voucher specimen was made and placed in the institutional herbarium to be referred to later. The retrieved leaves were washed with distilled water to discard any debris and dried over four days in a hygienic environment, and then processed.

2.2 Extraction Procedure

The powdering of the dried leaves was done by a mechanical grinder and then stored in airtight containers. Approximately 100 g of the powdered sample was exposed to successive Soxhlet extraction with hexane, ethyl acetate, ethanol and distilled water solvents with increasing polarity. All the extractions took 12h to completely extract the phytoconstituents. Reduced pressure rotary vacuum evaporator was used to concentrate the obtained extracts and vacuum-dry. The dried extracts were placed in the 4 °C sterile containers awaiting analysis (Figure 1).

2.3 Phytochemical Screening And Gc–Ms Analysis

To determine the major secondary metabolites in all extracts, preliminary phytochemical screening of all extracts was conducted with the help of certain common qualitative tests: the presence of alkaloids, flavonoids, phenolics, saponins, terpenoids and steroids. The resulting ethanol extract, which contained a great deal of phytochemical variety, was then subjected to analysis by GC-MS with the aim of identifying the different compounds. On the basis of the retention time, molecular weight and mass spectral fragmentation patterns, 27 phytoconstituents have been identified and correlated against standard reference libraries like NIST (Table 1). GC–MS analysis was performed using a capillary column and electron impact ionization mode at 70 eV. Identification of compounds was achieved by comparing mass spectra with the National Institute of Standards and Technology (NIST) spectral library. Only compounds with a similarity index greater than 90% were considered for identification. The chemical structures, molecular formulae and retention times of the identified phytoconstituents were recorded.

2.4 Bioinformatics Workflow

This analysis of the 27 compounds identified in the ethanol extract was done through in-silico drug-like screening through the SwissADME web server. Such parameters as Lipinski rule of five, gastrointestinal absorption, and blood-brain barrier (BBB) permeability were tested. On these grounds, nine compounds exhibiting desirable pharmacokinetic characteristics and large BBB permeability were chosen.

Target prediction of the ligands of choice was performed on the SwissTargetPrediction and PharmMapper servers. Targets related to the disease were gathered in disease-specific target databases. The Venny tool showed the intersection of the predicted targets of the ligands and disease targets, and 355 overlapping targets were identified.

The Complex interaction pattern was visualised by creating a compound target interaction network through the Cytoscape software. Protein-protein interaction (PPI) and network topological parameters analysis were conducted through the STRING database, and the identified hub genes were AKT1, CASP3, GSK3B, and MAPK1. The analysis of functional enrichment was performed with the help of ShinyGO, including Gene Ontology (GO) and KEGG pathway analysis. The PI3K Akt signalling pathway was also reported as a significant neuroprotective regulatory pathway.

2.5 Molecular Docking

In order to confirm the affinity of the identified nine compounds against the most important neurodegenerative targets, molecular docking studies were conducted. The target protein structures were Akt1 (PDB ID: 3CQW), GSK-3β (PDB ID: 4ACG), and β-amyloid (PDB ID: 1IYT). Molecular docking, Docking was done through AutoDock or similar software. The stability and inhibitory potential of the ligand-target complexes were assessed through the binding energies, hydrogen bond and binding conformations.

2.6 Cell Line And Culture Conditions

In vitro studies were done using the human neuroblastoma SH-SY5Y cell line. Cultured cells were in Dulbecco modified Eagle medium (DMEM) that was supplemented with L-glutamine, sodium bicarbonate, and antibodies (penicillin, streptomycin and amphotericin B). The cultures were left in a humidified 5% CO_2 incubator at 37 °C.

2.7 Mtt Cell Viability Assay

In the determination of the cytoprotective and neuroprotective properties of Alstonia venetata extract against neurotoxicity caused by $AlCl_3$, the MTT assay was conducted. The cells were subjected to different levels of the extract (25-400 µg/mL) after the treatment with aluminium chloride. The plates were incubated through the incumbency of 28 days. The spectrophotometric measurement was done to measure cell viability, and the IC_{50} value was computed to establish the effective neuroprotective concentration.

2.8 Caspase-3 Apoptosis Elisa

The percentage of apoptosis was determined by estimating the caspase-3 activity using the ELISA technique. The cells were incubated with 100 µM AlCl3 after 24 h, and then this was extracted. Specific activity of caspase was calculated by the optical density at 415 nm, and estimation of the protein concentration was done based on the Bradford method.

2.9 AKT Expression Studies by Flow Cytometry

A cell suspension of 2ml containing ~2.5 x 105 cells per well, was seeded into the 6 well plate without any test agent. The cells were incubated at 37 °C in 5% CO_2 atmosphere for approximately 24 hours to ensure proper attachment and growth.After 24hrs, cells were stimulated with insulin (100nM) and treated with wortmannin, test compound, or test compound + wortmannin according to the study design.At the end of treatment, the culture medium was aspirated, and the cells were washed with PBS.At the end of the treatment, the medium was aspirated from all the wells and given a PBS wash.200µl of trypsin-EDTA solution was added and incubated at 37°C for 3-4 minutes. 2 ml culture medium was added and the cells were harvested directly into 12 x 75 mm polystyrene tubes.The cells were centrifuged at 300 × g for 5 min at 25 °C, and the supernatant was carefully discarded.The cell pellet was washed with PBS and fixed by adding 1 mL of cold 70% ethanol dropwise while vortexing gently, followed by incubation for 30 min at −20 °C.

Note: Add dropwise to cell pellet while vortexing. This should ensure fixation of all cells and minimize clumping. Fixed cells were pelleted by centrifugation at a higher speed for 5 min, the supernatant was aspirated, and the cells were washed twice with PBS.10 µL of FITC-conjugated antibody was added to the cell pellet, mixed thoroughly, and incubated for 30 min in the dark at room temperature (20–25 °C).The cells were washed with PBS to remove unbound antibody and resuspended in 500 µL PBS.The cells were mixed thoroughly and analyzed by FACS using the FL1 channel

2.10 Standardization of Extract

The ethanol extract was chemically characterized using GC–MS and standardized based on the relative abundance of major phytoconstituents including β-sitosterol, squalene and quinic acid.

3. Results

3.1 Phytochemical and Ligand Screening Results

The analytical results of the ethanol extract of Alstonia venenata by GC-MS analysis showed that the extract contained 27 phytochemical compounds, which is a high concentration of bioactive elements. SwissADME was also used to screen these compounds for drug-likeness. Out of these, nine of them met the Lipinski rule of five, were highly gastrointestinally absorbed and had good blood-brain barrier (BBB) permeability, indicating their potential in neuroprotective use. Nine of these ligands were chosen in order to provide a more in-depth prediction of targets, network pharmacology, and molecular docking (Table 2). The chemical structures and molecular formulae of the major identified phytoconstituents are presented in Table 2.

3.2 Target Prediction and Network Analysis

Prediction procedures of the nine ligands of interest through the SwissTargetPrediction and PharmMapper methods provided target predictions of the selected 9 ligands to yield a total of 1138 possible protein targets after the elimination of duplicates. The genes associated with AD were retrieved in the target databases of diseases and were intersected with the ligand-predicted targets. The Venn diagram in Figure 2 revealed that there were 355 overlapping targets that were common, and this makes the therapeutic value high. The description of the complexity of the ligand-protein interaction made a compound-target interaction network in Cytoscape using these overlapping targets, consisting of 351 nodes and 3791 edges as shown in Figure 3. The high network structure shows the polypharmacological characteristic of Alstonia venetata phytoconstituents against neurodegenerative pathways.

3.3 Protein–Protein Interaction (PPI) Network and Hub Gene Identification

These 355 overlapping targets were then analysed further through protein interaction (PPI) through the STRING database. The built PPI network was highly enriched ($p < 1.0 \times 10^{-16}$) and sufficiently connected in the topology. The network topology analysis revealed the presence of a few hub genes, which include: AKT1, CASP3, STAT3, MAPK1, EGFR, GSK3B, and GAPDH as shown in Table 3 and Figure 4. They play important regulatory roles in neuronal survival, apoptosis, oxidative stress and intracellular signalling, which confirm their significance in the pathogenesis of AD and neuroprotection(Table 3).

3.4 Gene Ontology (GO) Enrichment Results

The analysis of gene ontology showed a significant clustering of 355 target genes into important biological processes, molecular functions and cellular components. Regulation of programmed cell death, apoptotic processes, cellular response to stress and chemical stimulus appeared to be the most enriched biological processes, and this is an indication that the mechanism has a strong implication in the neurodegenerative process. ATP binding, protein kinase, enzyme binding, and catalytic functions were the predominant molecular functions, indicating that the genes were involved in the adaptation to the phosphorylation and survival signalling pathways. The general localisation patterns of cellular components mainly

revealed a significant enrichment of synaptic distant, membrane rafts and caveolae as well as membrane microdomains that are involved in neuronal signalling and neurotransmission.
The dot plot in Figure 5 demonstrates the hugely enriched biological processes according to the values on the -log10(FDR). The strongest processes are cellular response to lipid, cell response to organic cyclic compounds, programmed cell death regulation, apoptotic process, intracellular signal transduction and a response to stress. The size of the dot is proportional to the number of genes taking part in every process, which illustrates that the identified targets are highly associated with apoptosis, oxidative stress, and neurodegenerative processes.
The plot in Figure 6 shows the enrichment of molecular functions like enzyme binding, protein kinase activity, ATP binding, kinase binding, catalytic activity on a protein and phosphotransferase activity. The categories show functional roles of identified genes as core elements in identifying some essential processes as signal transduction, phosphorylation, and cellular survival, which are important to neuroprotective signalling.
The analysis in Figure 7 indicates that the differentially expressed genes are mostly localised in caveolae, plasma membrane rafts, membrane microdomains, synapses, mitochondria, receptor complexes and cell junctions. These increased components of cells play a crucial role in neuronal communication, membrane signalling, and synaptic transmission, and these targets are involved in the modulation of neurodegenerative diseases.

3.5 KEGG Pathway Analysis
The analysis of the overlapping targets revealed that cancer pathways were the most predominant, followed by insulin resistance, lipid and atherosclerosis, MAPK signalling, and PI3Kingestase pathways through KEGG pathway enrichment analysis. Out of them, the PI3K10 Akt signalling pathway turned out to be the most prevalent neuroprotective one, and 10/ 20 core genes were involved in neuronal survival and prevention of apoptosis(Figure 8).
Figure 8 shows that AKT1 is a key regulator of various downstream signalling cascades of cell survival, death inhibition, metabolism, protein synthesis, and cell division.

3.6 Molecular Docking Results
The molecular docking study revealed that the selected ligands had high binding affinities with the important Alzheimer's disease targets of Akt1, GSK-32 and 2-amyloid proteins. Squalene, sitosterol, as well as quinic acid had the highest stable binding interactions with low energy binding scores in the results. These interactions suggest that they have a high inhibitory potential on amyloid aggregation, tau hyperphosphorylation and apoptosis-related signalling (Table 4).

3.7 MTT Cell Viability Results
The MTT test revealed a dose-related neuroprotective response of Alstonia venenata extract on AlCl3-induced toxicity in SH-SY5Y cells. The IC 50 was found to be in the range of 51.5 µg/ml. Moderate protection was provided by lower concentrations (25-50 unknown µg/mL), and higher concentrations (100400 µg/mL) had significant and increased effects on cell viability. A high percentage of viability of the cells was noted at 400 µg/mL, with 95 percent, which supports high cytoprotective ability.The bar graph in Figure 9 represents the dose-response protective effect of the Alstonia venenata leaf extract against the neurotoxicity of aluminium chloride on SH-SY5Y cells.

3.8 Caspase-3 ELISA Results
Caspase-3 activity in AlCl3-treated cells was significantly increased, which proved increased apoptosis. A combination of Alstonia venenata extract with another medicinal substance (Caspase-3) has considerably lowered the caspase-3, thus showing a high anti-apoptotic neuroprotective activity.
The bar graph in Figure 10 indicates that caspase-3 activity is high in the AlCl3-treated group, which is a confirmation of the induction of apoptosis. Caspase-3 activity was significantly mitigated to close to control under co-treatment with Alstonia venenata, thus depicting a powerful anti-apoptotic and neuroprotective effect (Table 6).

3.9 AKT Expression Studies by Flow Cytometry
The effect of test compounds on AKT expression in SHSY5Y cell lines was analysed by flow cytometry. The results indicated that the test compound suppresses the effect of the inhibitor on insulin-stimulated AKT expression in SHSY5Y cells(Fig 11 and 12). The sample increased the percentage of cells showing the AKT pathway compared to the inhibitor wortmannin alone.

4. Discussion
4.1 Significance of Multi-Target Neuroprotection
The amyloid-beta aggregation, oxidative stress, neuroinflammation, mitochondrial dysfunction, and neuronal apoptosis are present in the pathogenesis of amyloid-beta in AD, which causes cognitive decline and progression [25,26]. Compared to single-target therapies, multi-target neuroprotection is a key to ensuring the disease progression is stopped, and it is urgent to address these pathological pathways that are coupled to each other [26,27]. The network pharmacology findings in the present study highly support this multi-target concept because they identified 355 overlapping targets between Alstonia venetata phytoconstituents and AD-associated genes and a dense protein-protein interaction network with 351 nodes and 3791 edges.

Apoptosis, oxidative stress and important signalling pathways through Nrf2, MAPK or cannabinoid receptor signalling can be modulated using natural extracts and multi-target drugs, which have synergistic neuroprotective properties [28-30]. Consistent with this idea, the GO and KEGG enrichment analyses identified that there was a robust regulation of apoptotic processes, stress-response signalling, kinase activity, and PI3KAkt signalling that are highly coregulated in neuronal survival. In addition, the presence of major hub genes in our PPI analysis (AKT1, CASP3, MAPK1, GSK3B, and EGFR) has indicated that this plant (Alstonia venenata) can regulate both cell survival and apoptotic cell execution pathways at the same time.

Quercetin and astaxanthin, among other compounds, have been shown to reduce the oxidative damage, amyloid aggregation and neuroinflammation to preserve the neuronal functions [29,31]. Likewise, in the current research, molecular docking revealed to have high binding affinities of β-carotene, sitosterol, squalene, and quinic acid with Akt1, GSK-3 and 8, and with 2-amyloid, which suggests that they will not restore their potential interference with multiple pathological targets of AD.

Others that are also found in multi-target methods encompass enzyme-inhibition, as in acetylcholinesterase, and control of metal ion-induced toxicity, which are all part of the AD pathology [25,32]. It is interesting to note that the model of neurotoxicity caused by AlCl3 is directly proportional to the neurotoxicity caused by the same metal. The fact that Alstonia venenata will rectify the oxidative cytotoxicity and apoptosis is further an indication of its suitability as a multi-target neuroprotective agent. On the whole, the combination of network analysis of pharmacology, molecular docking, and neuroprotective activity in the current study is strong evidence of the idea that multi-target neuroprotection extends to curing AD compared to administering single-target medications [26,27,30].

4.2 Role of PI3K-Akt Pathway in Neuroprotection

The PI3K-Akt signalling pathway has one of the most vital roles in neuroprotection through attenuating apoptosis and enhancing neuronal survival. Activation of Akt results in the inhibition of pro-apoptotic proteins (Bax and caspase-3), accompanied by the upregulation of anti-apoptotic proteins (Bcl-2 and Bcl-xL), which finally mitigates neuronal cell death during ischemic and neurodegenerative disorders [33-35]. Notably, the appropriateness of this pathway was effectively reinforced in the current study, with KEGG pathway enrichment analysis contributing to the most dominant neuroprotective pathway, which was found to be PI3K–Akt signalling pathway; consequently, several targets overlapped by this cascade were placed. Moreover, AKT1 was one of the central hub genes in the protein-protein interaction network, which validated AKT1 as a central regulatory point in the observed multi-target neuroprotective mechanism.

Akt also suppresses the activity of glycogen synthase kinase-3B (GSK-3B), which decreases tau protein hyperphosphorylation as one of the pathological changes characteristic of Alzheimer's disease [36,37]. Considering this mechanism in very high regard, this study has shown, using the molecular docking experiments, high-binding affinity of 0-carotene, sitosterol, 0-linolenic acid, squalene, and quinic acid with Akt1 and GSK-3 8, indicating that these phytoconstituents of Alstonia venenata can directly disrupt the signalling mechanism of apoptosis and tau.

4.3 Interpretation of Docking and In-Vitro Correlation

The high binding affinities of major Alstonia venenata phytoconstituents to Akt1 and GSK-32, found in the molecular docking experimentation, are a clear-cut mechanistic foundation to the neuroprotective impacts observed in the in-vitro tests. Akt1 had a high affinity to compounds like 2-carotene, sitosterol, squalene, quinic acid and alpha-linolenic acid, indicating that the survival signal pathway was activated. This high interaction of Akt is directly related to the high degree of inhibition of activity of caspases-3 measured in the ELISA assay, and thus apoptosis inhibition. Also, the observed dose-dependent amplification of cell viability of the MTT assay (up to the range of 95 per cent) is consistent with the proposed Akt-mediated cytoprotective signalling. These results combined contribute to a good docking in vitro mechanism correlation, which proves the PI3KAktcaspase axis to be the key neuroprotective pathway of Alstonia venetata. These findings suggest that specific phytoconstituents such as β-sitosterol, squalene and quinic acid may represent the principal neuroprotective agents present in the extract.

4.4 Comparison with Previous Alstonia Studies

Most of the earlier investigations of Alstonia have primarily reported its antidepressant and antiepileptic activity, and it has the potential for usage in the treatment of mood disorders and also in seizure-related illnesses. The articles concentrate on the symptom relief and safety of antidepressants and antiepileptic drugs, and in many cases, it is recommended that additional clinical trials are needed to determine their efficacy and mechanisms of action [38-40]. The new value of the recent work is the opportunity to use Alstonia in the Alzheimer's disease (AD) model, and the bioinformatics tools are used to investigate the molecular pathways, especially the apoptotic ones. This integrative strategy goes beyond symptomatic treatment to find out the effects of Alstonia in neurodegenerative processes to provide new information on the neuroprotective mechanisms of Alstonia. Integrating experimental AD models with computational studies, the study contributes towards the comprehension of Alstonia in the management of AD, an area that has not been studied before in the literature.

4.5 Strengths and Limitations

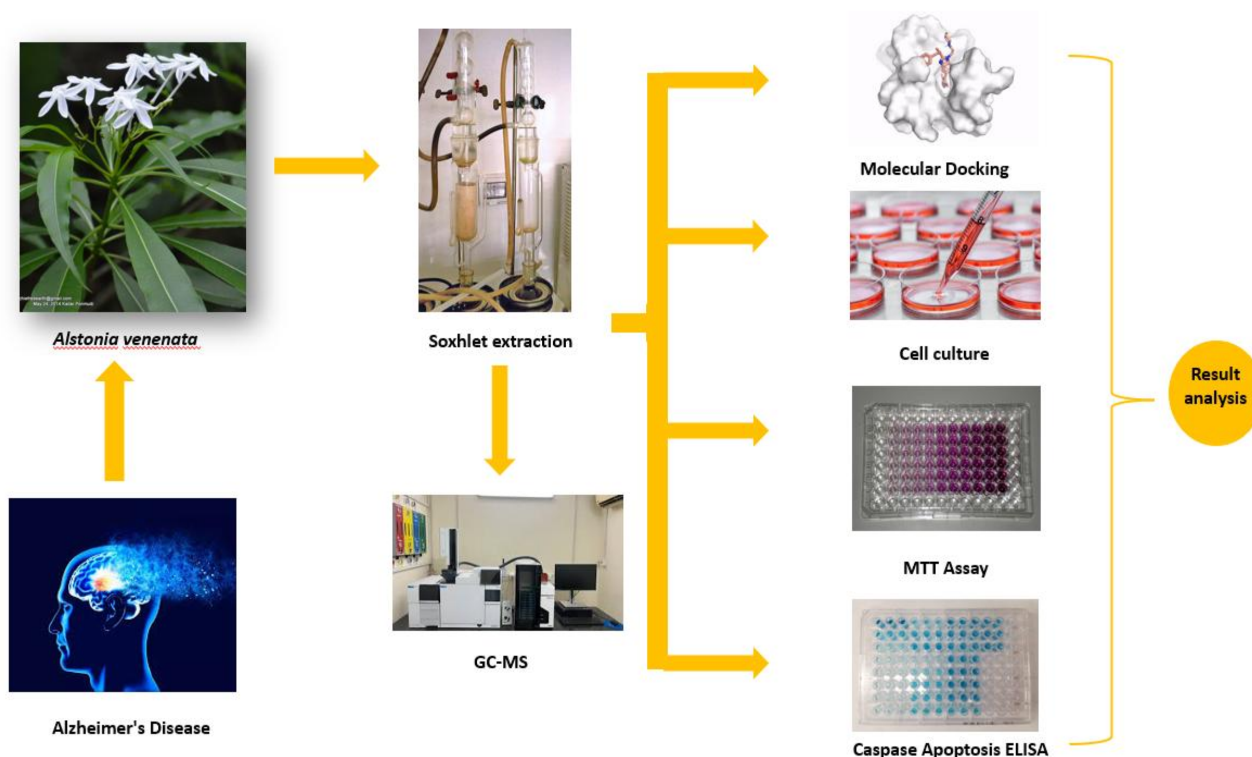
One of the strengths of the current study is that it uses a triple level of validation, which involves in-silico network pharmacology and molecular docking and in-vitro neuroprotective assay, a methodology that puts high mechanistic validity on the results. It is suggested that the complex nature of Alzheimer's disease is well-explained with the help of the multi-target strategy that allows this research to enhance the therapeutic applicability of Alstonia venenata. The study, however, has limitations in the fact that they have not been confirmed in vivo, and behavioural and cognitive testing have not been done. It is necessary that future research into the use of animal models and profiling of behaviour should identify and validate the translational nature of these findings.

5. Conclusion and Future Scope

The current research can be well supported by the finding that Alstonia venenata has a powerful neuroprotective effect in a multi-target mechanism. The use of combined network pharmacology and molecular docking studies found that bioactive phytoconstituents of A. venenata could regulate the PI3K-Akt-GSK-3 -caspase sequence, which is part of the neuronal survival and apoptosis regulation pathway [36]. These in-silico results were experimentally confirmed using MTT and caspase-3 ELISA tests, which showed that the AlCl 3-induced apoptosis was significantly reduced and that cell survival by SH-SY5Y neurons was significantly improved. The existence of the correlation between Akt activation and caspase-3 inhibition is another factor that substantiates the mechanistic importance of the PI3K Akt pathway [26,33]. Overall, these findings indicate that the identified phytoconstituents, particularly β-sitosterol, squalene, quinic acid and α-linolenic acid, may serve as potential lead molecules for future anti-Alzheimer drug development.

Future studies should hence concentrate on evaluation of biological effects in vivo by using proper Alzheimer disease animal model, measurement of behaviour and cognitive effects, blood-brain barrier pharmacokinetics profiling, isolation and structural characterisation of lead neuroprotective phytoconstituents to aid in preclinical development.

Graphical Abstract



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Abbreviations

AD - Alzheimer’s disease

GC-MS -Gas Chromatography -Mass spectroscopy

ELISA assay- Enzyme -linked immunosorbent assay
MTT assay- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay
GO -Gene ontology
IC 50 - inhibitory concentration 50
EGFR- Epidermal Growth Factor Receptor
MAPK- Mitogen Activated Protein Kinase
CASP- Caspases
STAT3- Statin
PDB -Protein Data Bank
CNS-Central Nervous System
AlCl3 -Aluminium Chloride

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TABLES

Table 1: Qualitative Phytochemical Screening of Alstonia venenata Leaf Extract

Sl. No.	Phytoconstituents	Test Performed
1	Carbohydrates	Fehling's solution test
2	Proteins and amino acids	Xanthoprotein test, Ninhydrin test
3	Alkaloids	Dragendorff's test, Hager's test
4	Steroids and Triterpenoids glycosides	Salkowski test
5	Cardiac glycosides	Keller–Killiani test
6	Oils and fats	Spot test
7	Phenolic compounds	Ferric chloride test
8	Terpenoids	Noller's test
9	Tannins	1% Lead acetate test
10	Glycosides	Anthrone + Sulphuric acid test
11	Coumarins	10% NaOH test
12	Saponins	Foam test
13	Flavonoids	Ferric chloride ($FeCl_3$) test
14	Quinones	H_2SO_4 test
15	Starch	I_2KI_2 test
16	Phenols	Ferric chloride ($FeCl_3$) test
17	Gum	Water test

Table 2: SwissADME-Based Drug-Likeness and Pharmacokinetic Properties of Selected Bioactive Compounds from Alstonia venenata

SL No.	Molecule	Molecular Formula	MW (g/mol)	Rotatable Bonds	H-Bond Acceptors	H-Bond Donors	TPSA (Å²)	GI Absorption	BBB Permeant	Lipinski Violations	Bioavailability Score
1	p-Hydroxymethyl furfural	$C_6H_6O_6$	126.11	2	1	1	20.23	High	Yes	0	0.55
2	β-Amyrin	$C_{30}H_{50}O$	426.73	6	2	1	35.25	High	Yes	0	0.55
3	Quinic acid	$C_7H_{12}O_6$	192.17	6	5	1	57.15	High	Yes	0	0.55
4	α-Linolenic	$C_{18}H_{30}O_2$	278.43	5	3	0	35.53	High	Yes	0	0.55

	acid										
5	β-Sitosterol	$C_{29}H_{50}O$	414.72	4	5	1	64.99	High	Yes	0	0.85
6	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	14	2	1	37.30	High	Yes	0	0.85
7	Pentadecanoic acid	$C_{16}H_{32}O_2$	256.40	13	2	1	37.30	High	Yes	0	0.85
8	Squalene	$C_{30}H_{50}$	410.72	15	0	0	0.00	High	Yes	0	0.85
9	Carotene	$C_{40}H_{56}$	536.87	0	3	3	45.67	High	Yes	0	0.56

Table 3: Major Hub Genes Identified from the PPI Network

Sl. No.	Hub Gene	Biological Role
1	AKT1	Cell survival signaling
2	CASP3	Apoptosis execution
3	GSK3B	Tau phosphorylation
4	MAPK1	Stress signaling
5	EGFR	Growth factor signaling
6	STAT3	Transcription regulation

Table 4: Molecular Docking Binding Energy Scores (kcal/mol) of Selected Bioactive Compounds from Alstonia venenata Against Alzheimer's Disease Target Proteins.

Sl. No.	Compound Name	Akt/PI3K (3CQW)	GSK-3β (4ACG)	β-Amyloid (1IYT)
1	Hydroxymethyl furfural	−2.948	−5.825	−1.607
2	β-Carotene	−8.302	−6.175	−7.345
3	Squalene	−6.784	−5.022	−6.314
4	Quinic acid	−6.380	−5.111	−5.234
5	Sitosterol	−7.543	−6.008	−6.378
6	Hexadecanoic acid	−3.987	−3.958	−3.940
7	β-Amyrin	−4.060	−3.264	−2.319
8	α-Linolenic acid	−6.987	−5.998	−4.659
9	Pentadecanol	−0.529	−2.119	−2.175

Table 5: Effect of Alstonia venenata Leaf Extract on Cell Viability in $AlCl_3$-Induced SH-SY5Y Cells (MTT Assay)

Treatment	Concentration (µg/mL)	Absorbance at 570 nm	Cell Viability (%)
Control	–	0.8000	100.0
Aluminium chloride ($AlCl_3$)	100 µM	0.4336	54.2
A. venenata + $AlCl_3$	25 µg/mL	0.5024	62.8
A. venenata + $AlCl_3$	50 µg/mL	0.5720	71.5
A. venenata + $AlCl_3$	100 µg/mL	0.6632	82.9
A. venenata + $AlCl_3$	200 µg/mL	0.7232	90.4
A. venenata + $AlCl_3$	400 µg/mL	0.7608	95.1

Table 6: Effect of Alstonia venenata Leaf Extract on Caspase-3 Activity in $AlCl_3$-Induced SH-SY5Y Cells (Indirect ELISA Method)

Treatment Group	Absorbance	Protein Concentration (mg/mL)	Caspase-3 Activity (Unit/mg Protein)
Control	0.3746	0.5469	0.6849
Aluminium chloride ($AlCl_3$)	0.7340	0.7538	0.9737
Aluminium chloride + Alstonia venenata	0.5320	0.7700	0.6906
Alstonia venenata alone	0.4538	0.6403	0.7000

FIGURES

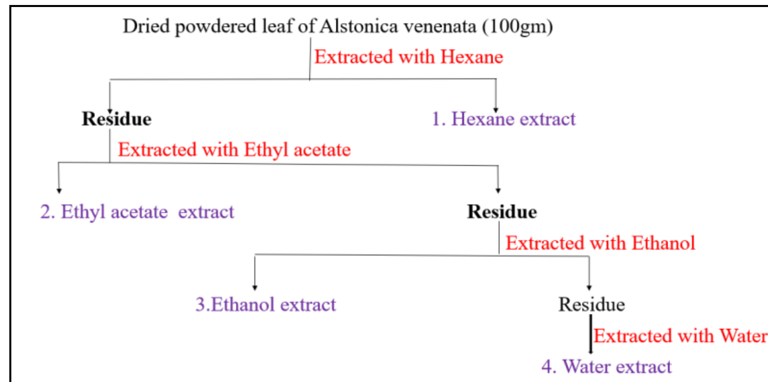

Figure 1: Solvent Extraction Process

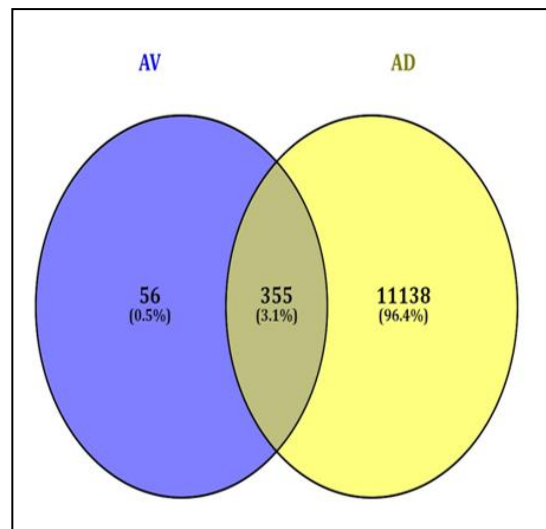

Figure 2: Venn diagram showing the intersection between Alstonia venenata (AV) target proteins and Alzheimer's disease (AD)-associated genes.

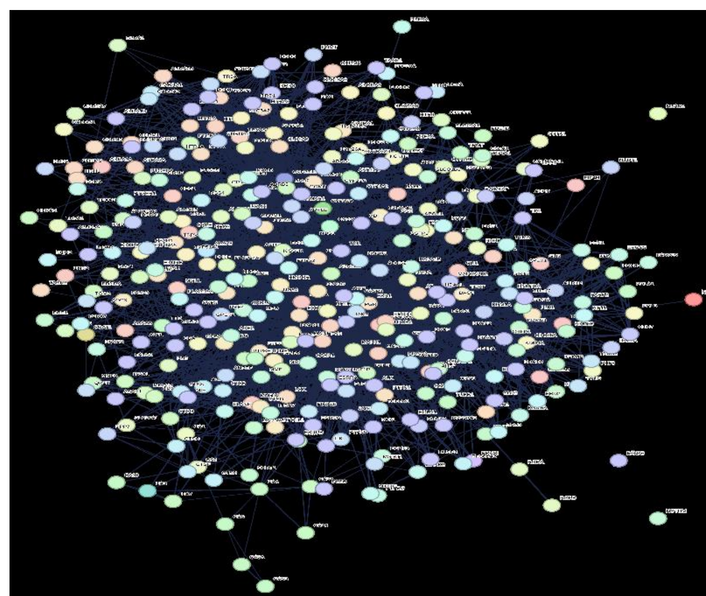

Figure 3: Protein–protein interaction (PPI) network of overlapping targets between Alstonia venenata and Alzheimer's disease constructed using the STRING database.

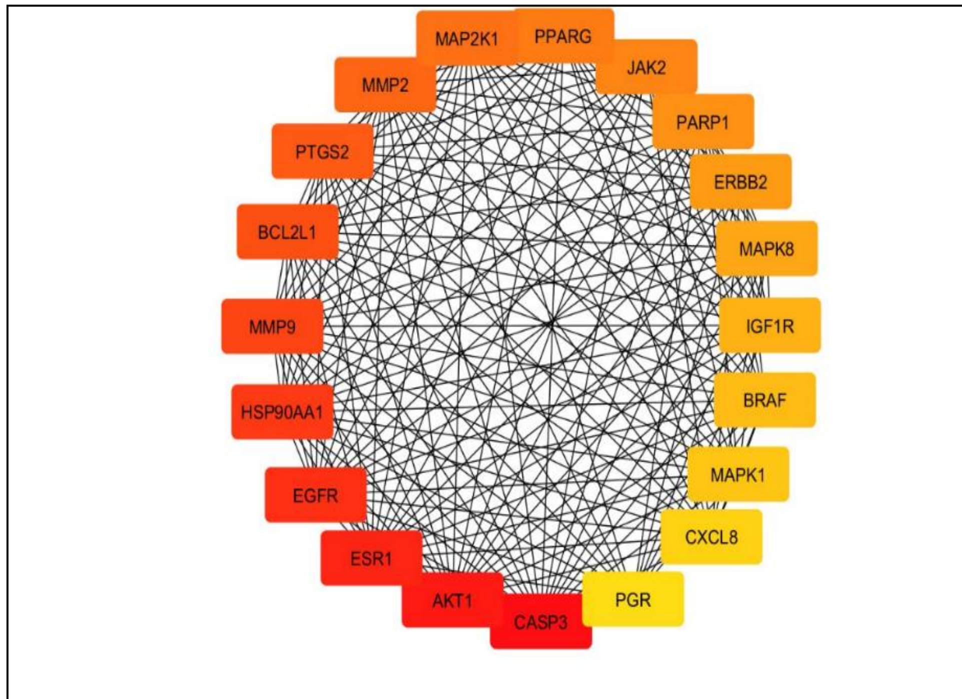

Figure 4: Hub gene interaction network of key regulatory targets involved in Alzheimer's disease generated using Cytoscape.

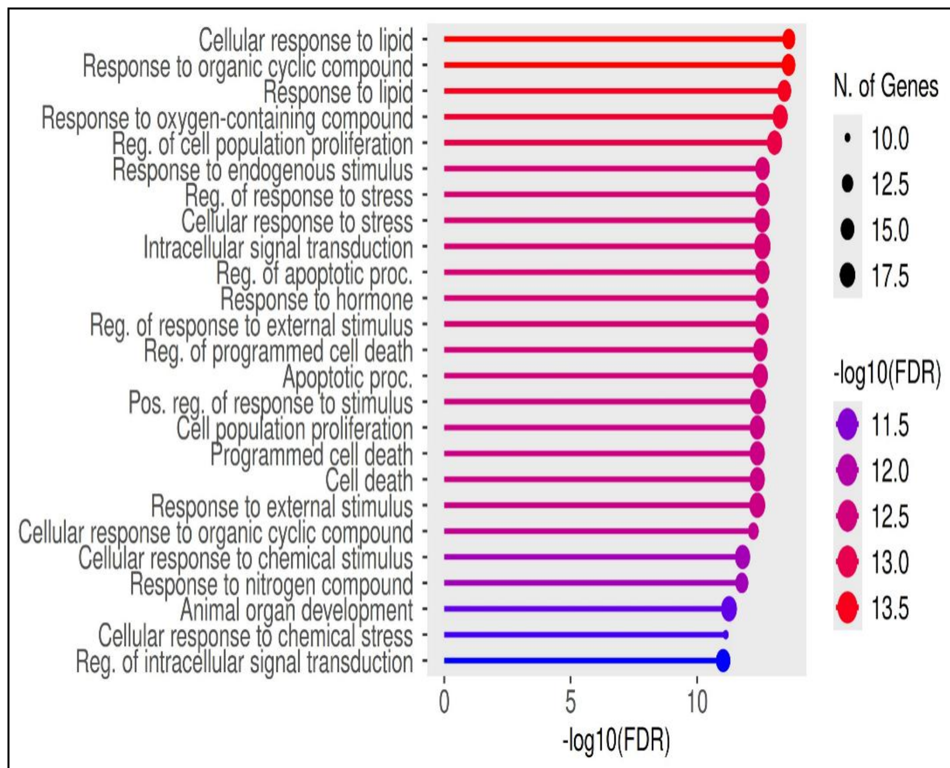

Figure 5: Gene Ontology (GO) Biological Process (BP) enrichment analysis of overlapping targets between Alstonia venenata and Alzheimer's disease.

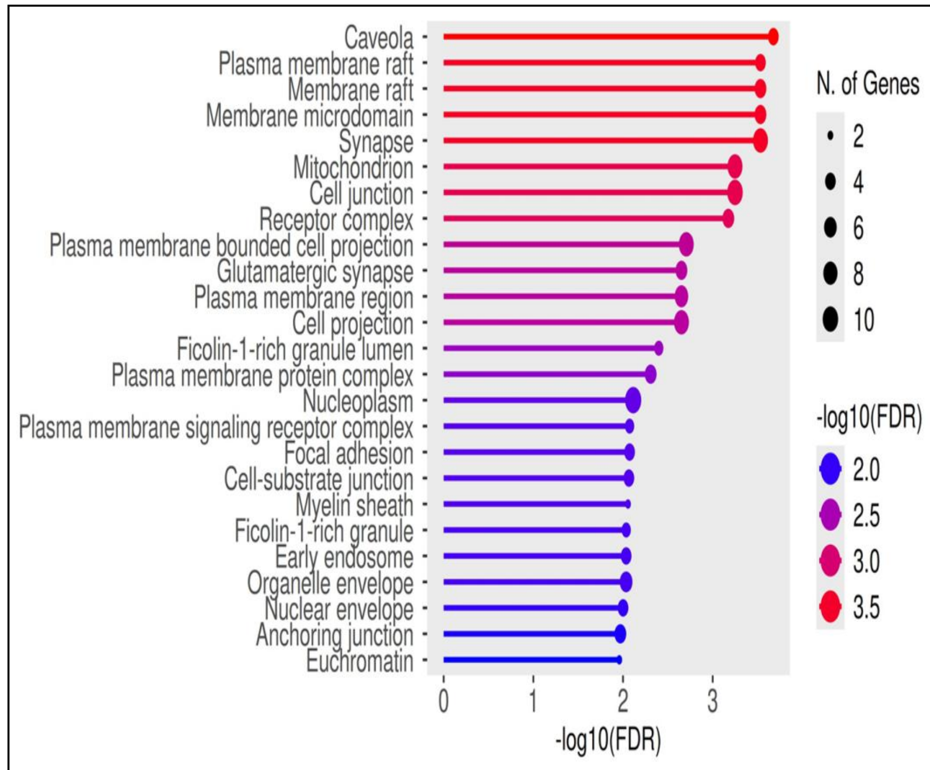

Figure 6: Gene Ontology (GO) Molecular Function (MF) enrichment analysis of the intersecting target genes.

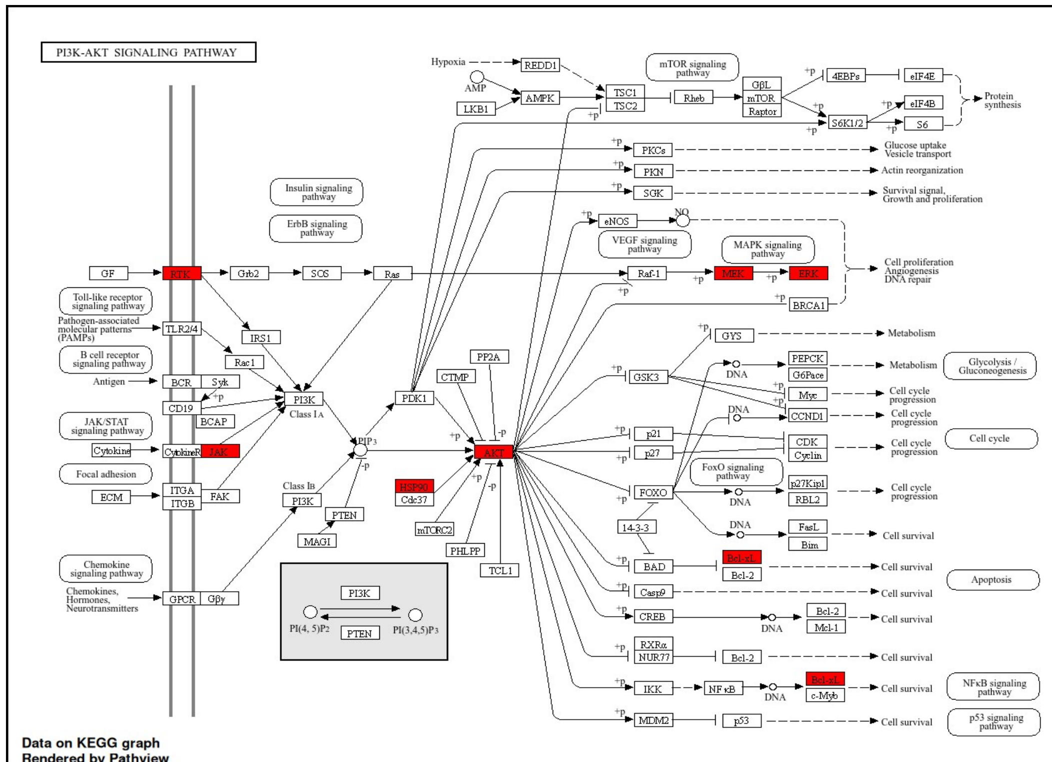

Fig. 7: Gene Ontology (GO) Cellular Component (CC) enrichment analysis of significant target genes.

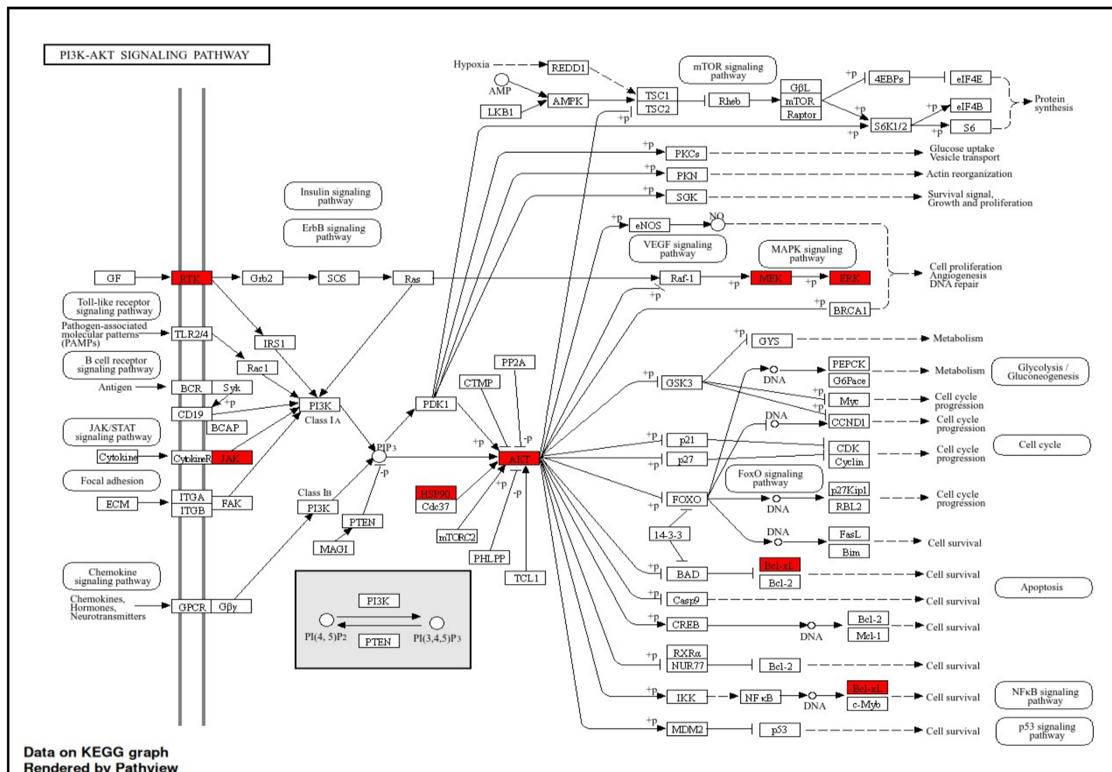

Figure 8: KEGG pathway map of the PI3K–Akt signalling pathway highlighting key target proteins modulated by Alstonia venenata phytoconstituents.

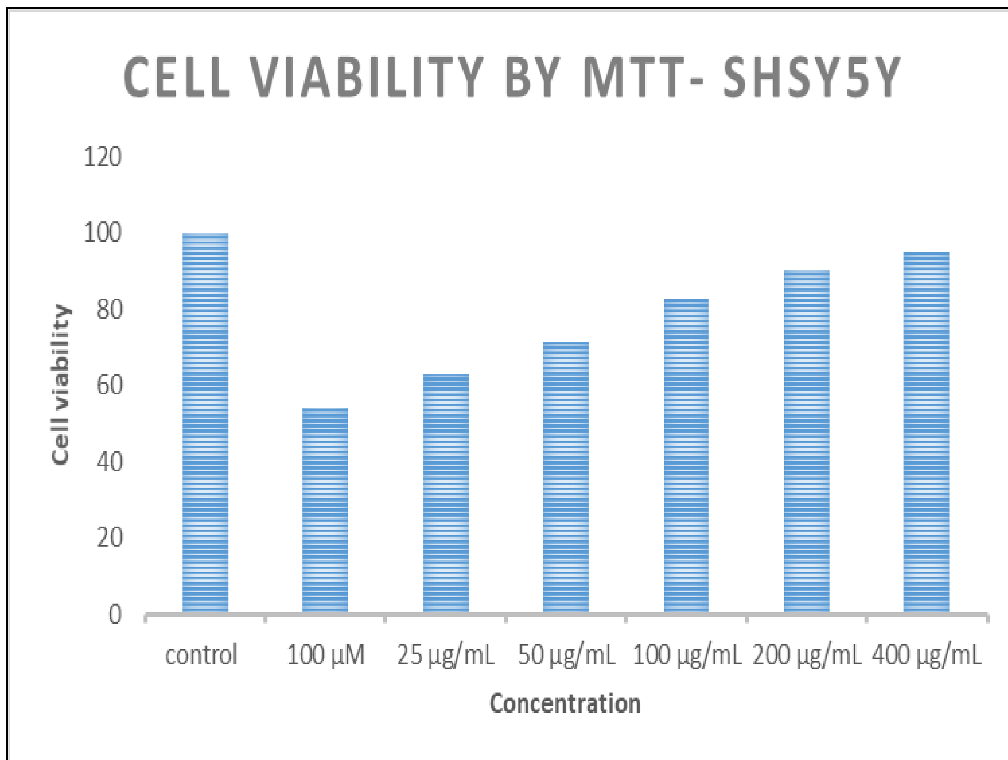

Figure 9: Effect of Alstonia venenata Leaf Extract on Cell Viability in $AlCl_3$-Induced SH-SY5Y Cells by MTT Assay.

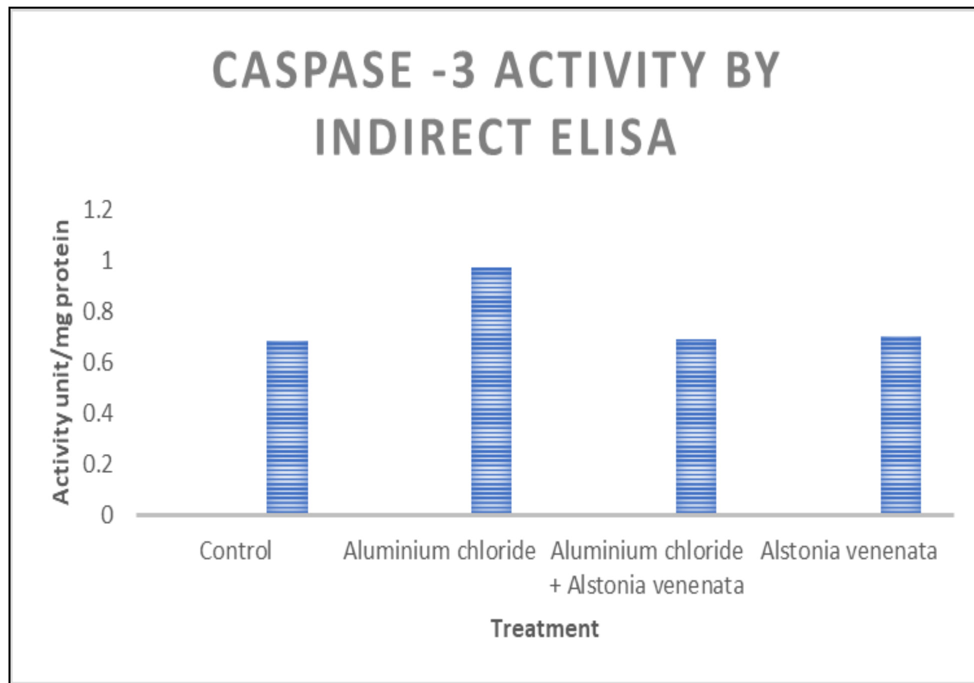

Figure 10: Effect of Alstonia venenata Leaf Extract on Caspase-3 Activity in $AlCl_3$-Induced SH-SY5Y Cells as Measured by Indirect ELISA.

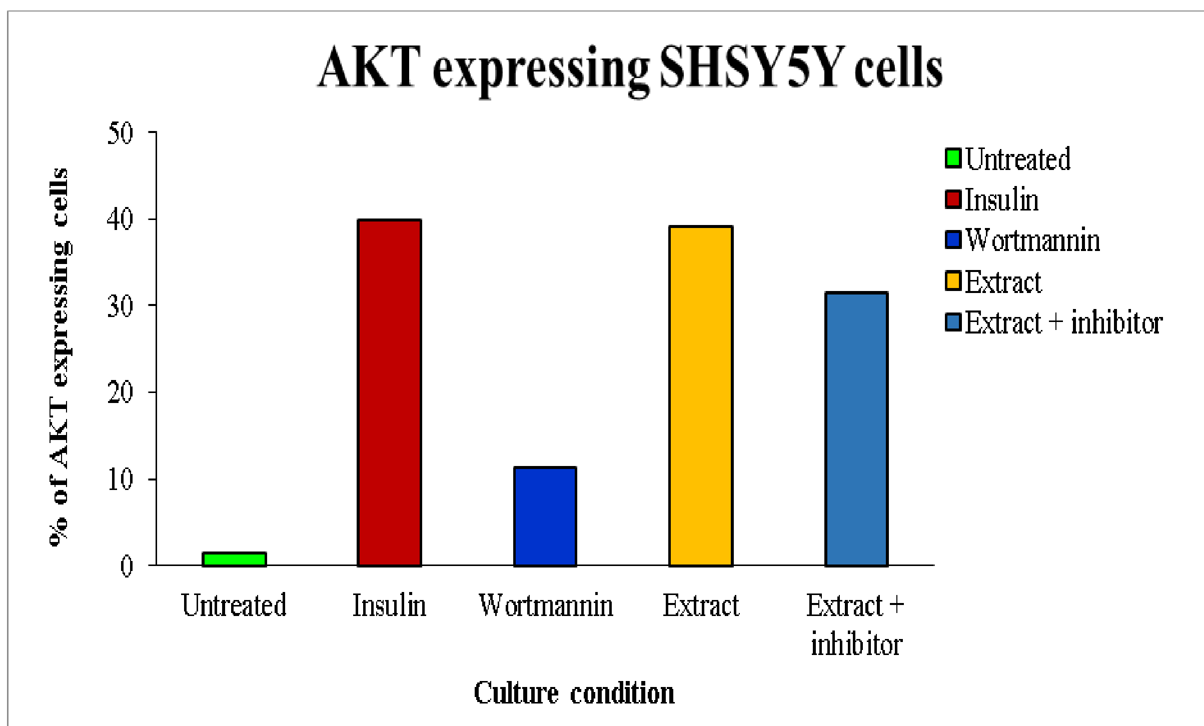

Fig 11. Percentage of SHSY5Y cells expressing AKT treated with the desired concentration of test compounds. SHSY5Y cells cultured without any drug treatment were used as the negative control.

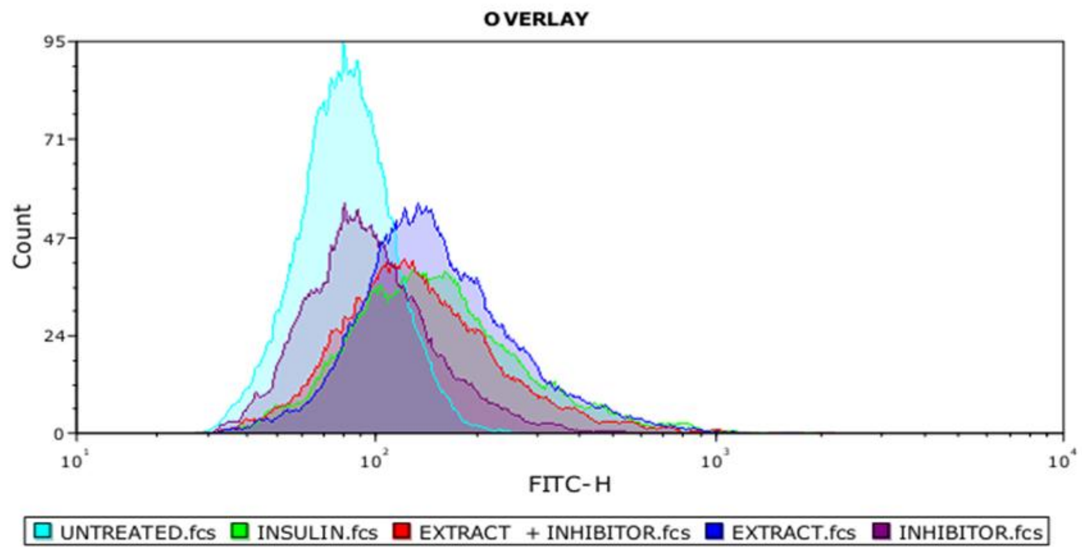

Fig 12 .Overlay showing FITC-channel fluorescence for different treatment groups in SH-SY5Y cells.