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NATURAL NEUROPROTECTION: INVESTIGATING THE EFFECTS OF *SOLANUM NIGRUM* EXTRACT ON ATP-INDUCED CELL PROLIFERATION IN PC12 CELLS

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Abstract

The significance of natural products in drug discovery has increased substantially, prompting intensified efforts to screen for potent drugs from natural sources. *S. nigrum*, commonly known as black nightshade, is documented to possess anti-proliferative, antioxidant, antimicrobial, and anti-inflammatory properties. This study aimed to investigate the impact of an extract derived from *S. nigrum* on ATP-induced cell proliferation in the PC12 cell line. Our findings demonstrate that exposure to *S. nigrum* (30 μM) led to a decrease in cell proliferation and an increase in cell death, as validated by cell counting, LDH assay, and H33258 staining. However, *S. nigrum* (30 μM) also significantly mitigated the harmful effects of ATP (100 μM) on the PC12 cell line. The outcomes of this research suggest that administering *S. nigrum* extract to the PC12 cell line for 72 hours can confer neuroprotective benefits. While the detailed impact of *S. nigrum* on the calcium-mediated pathway is still being analyzed, our preliminary data indicate its potential as an effective neuroprotective agent.

Key words: PC12, *Solanum nigrum*, Cytotoxicity, ATP, Neuroprotective.

Introduction

The significance of natural products in drug discovery has gained immense attention. Investigating plant extracts for novel drug discovery is a common practice (Atanu *et al*., 2011). *Solanum nigrum*, commonly known as black nightshade, is a dicot weed from the Solanaceae family, which includes approximately 84 genera and 3000 species. Solanum is one of the largest and most important genera (Nasir *et al*., 1981). Although several species in the Solanaceae family can be harmful to humans and animals, *Solanum nigrum* is considered safe for consumption. However, it contains solanine, a glycoalkaloid that can be toxic in certain doses, with toxicity levels varying depending on the amount consumed (Glossman-Mitnik, 2007). *S. nigrum* contains several compounds responsible for its diverse actions, such as polysaccharides, glycoproteins, glycol-alkaloids, and polyphenolic compounds like rutin, catechin, gallic acid, caffeic acid, proto-catechuic acid, naringenin, and epicatechin (Wannang *et al*., 2008). The chemical composition of plants plays a significant role in modern medicine, as they are used in various biological activities. *S. nigrum* has been investigated for its antiseizure (Lin *et al*., 2008), antioxidant, hepatoprotective (Bontempo *et al*., 2013), and antimicrobial (Lee *et al*., 2004) properties. The main active constituents of *S. nigrum* , glycoalkaloids and glycoproteins, exhibit anti-tumor activity (Li *et al*., 2009). The phytochemicals present in *S. nigrum* are recognized for their versatile properties. Studies have demonstrated that the crude extracts and isolated components of *S. nigrum* exhibit antiproliferative effects on various tumor cells. It has been reported that the release of Ca2+ from IP3 evokes ATP to induce an increase in intracellular free Ca2+ concentration, and the extracellular influx of Ca2+ stimulates P2Y-receptor-mediated phospholipase C (PLC), leading to enhanced formation of reactive oxygen species (ROS) (Brookes *et al*., 2004). Furthermore, extracellular ATP induces cell death in PC12 cells (Schulze-Lohoff *et al*., 1998; Sun & Chen, 1998). The neuroprotective effect of *S. nigrum* has also been investigated regarding the degeneration rate of motoneurons in rats (Gabrani *et al*., 2012). However, the effects of *S. nigrum* on ATP-induced cell death in PC12 cells have not been evaluated. The exploration of natural products for their therapeutic potential has become increasingly significant in drug discovery efforts. Among these, *Solanum nigrum*, commonly known as black nightshade, has garnered attention due to its diverse pharmacological activities and bioactive constituents. This plant, belonging to the Solanaceae family, is recognized for its safety in consumption despite containing potentially toxic glycoalkaloids like solanine (Chen *et al*., 2022). Recent studies have elucidated various beneficial properties of *Solanum nigrum*, including its antiepileptic and neuroprotective effects, antioxidative properties against cardiovascular stress (Alam *et al*., 2022), and a broad spectrum of bioactive components contributing to its medicinal potential (Chen *et al*., 2022; Zeeshan *et al*., 2023). These findings underscore the therapeutic promise of *Solanum nigrum* and warrant further exploration into its applications in modern medicine. This study explores the neuroprotective potential of *Solanum nigrum* extract against ATP-induced cytotoxicity in PC12 cells, focusing on its ability to inhibit apoptosis and promote cell proliferation. Unlike previous research, this work specifically evaluates the impact of *S. nigrum* on ATP-induced cell death, contributing to our understanding of its therapeutic potential in neuroprotection.

Material and Methods

Material was obtained as following: Horse serum (HS) (heat inactivated) from Invitrogen (Carsbad, CA, USA), DMEM (Dulbecco modified Eagle's medium), fetal bovine serum (FBS, heat inactivated) and other remaining chemicals from Sigma (St. Louis, MO, USA).

***S. nigrum* extract preparation:** To prepare the *S. nigrum* extract, ripe fruits of *S. nigrum* were collected from the surrounding locality of Sahiwal and washed thoroughly to remove dust and debris. Aqueous and ethanolic extracts were prepared from the fruits. The fruits were mashed and dipped into ethanol for 72 hours. The solvent was evaporated under reduced pressure, and the resulting dried extract was stored at −70ºC until analysis. The same procedure as mentioned in earlier reports (Chen *et al*., 2022; Zeeshan *et al*., 2023) was followed to prepare the aqueous extract.

Cell culture: PC12 cells, derived from rat pheochromocytoma and obtained from the American Type Culture Collection (ATCC), were cultured in 20 mm dishes in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % horse serum (HS) and 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 10% CO_2 and 90 % O_2. Cells from passages 6-14 were used for the experiments and were plated in six-well culture plates at a density of 3×104 cells per well. The cells were allowed to adhere and were used for experimentation after 2-3 days of plating.

Cell proliferation assay: To assess the impact of *S. nigrum* extract on cell proliferation, a trypan blue cell counting assay was conducted. The growth rate was evaluated as the percentage viability relative to vehicle-treated cells, which were considered 100% viable according to Phillips HJ's method from 1957. PC12 cells were initially seeded in 6-well plates at a density of 3×104 cells/well. After 24, 48, and 72 hours of continuous incubation, the number of cells was counted using a hemocytometer. Two researchers, who were blinded to the experimental design, took images of the cells under an inverted microscope. To calculate the number of cells, PC12 cells were trypsinized and the cell count was determined for cells treated with *S. nigrum* (30 µM).

Hoechst staining analysis for apoptosis: The current study utilized Hoechst 33258 staining to evaluate apoptosis in cells. Specifically, PC12 cells were seeded in 6-well plates at a density of 3×104 cells/well. The cells were exposed to *S. nigrum* (30 µM) for 24, 48, and 72 hours, after which they were fixed with 4% (w/v) paraformaldehyde and incubated with Hoechst 33258 (1 mg/mL) (Sigma) for 20 minutes at 37°C in the dark. The cells were then examined using an Olympus IX70 inverted fluorescence microscope. Randomly chosen fields were acquired from each treatment. The examination was conducted at 4°C for 30 minutes following treatment.

LDH assay: LDH release is commonly used as an indicator of membrane damage. In this study, a cytotoxicity detection kit from Roche (Mannheim, Germany) was employed to identify the release of LDH into the culture medium from the damaged cells. To measure the absorbance at 492nm, a multi-label counter system was utilized. On the other hand, to determine the maximum LDH release, all cells in the control wells were lysed by treating them with 1% Triton X-100 (Perkin Elmer, Boston, MA).

Statistical analysis

The means ± SEM for all cells were used to present the data obtained from multiple independent experiments. To determine the statistical significance, a paired Student's t-test was performed.

Results and Discussion

Anti-proliferative effect of *S. nigrum* on PC12 cells: In this study, we explored the effects of *Solanum nigrum* on the proliferation of rat pheochromocytoma cells (PC12). Both ATP and acetylcholine are known to mobilize intracellular calcium via P2Y1 receptor activation, impacting proliferation and DNA synthesis. We assessed PC12 cell proliferation using a trypan blue cell counting assay after 24, 48, and 72 hours of treatment with *S. nigrum* (30 µM) and ATP (100 µM). Our findings indicate that *S. nigrum* extract significantly inhibited ATP-induced proliferation in a time-dependent manner, suggesting its effectiveness in reducing proliferation in both the presence and absence of ATP. This study is the first to investigate the impact of *S. nigrum* on PC12 cells, and our results indicate a notable decrease in cell proliferation over time (Fig. 1).

***S. nigrum* ameliorate the ATP induced apoptosis of PC12 cells:** Previous research has shown that extracellular ATP can induce necrosis and apoptosis in mesangial and PC12 cells (Schulze-Lohoff *et al*., 1998; Sun and Chen, 1998). Using inverted phase contrast microscopy, we observed morphological changes in PC12 cells treated with ATP (100 µM) over 24, 48, and 72 hours. ATP treatment led to significant cell shrinkage and a morphological shift from polygonal to round shapes. However, co-treatment with *S. nigrum* (30 µM) mitigated these cytotoxic effects, and cells maintained healthier growth and morphology over the same time periods (Fig. 2).

Hoechst staining analysis for apoptosis: We utilized Hoechst 33258 dye to conduct a morphology-based analysis of apoptosis. PC12 cells treated with *S. nigrum* for 24, 48, and 72 hours were fixed and stained to observe changes in nuclear morphology. After 48 hours of ATP treatment, cells exhibited characteristics of apoptosis, including shrinkage and chromatin condensation. However, treatment with *S. nigrum* restored the cells to their original morphology (Fig. 3A & B).

Amelioration of ATP-Induced Cell Death by *S. nigrum*: *S. nigrum* itigates ATP-induced cell death: In the control group treated with the vehicle, cultured PC12 cells exposed to ATP (100 µM) for 24, 48, and 72 hours exhibited significant neuronal damage, as indicated by comparison with untreated cells using Hoechst 33258 dye. To evaluate *S. nigrum's* protective effect against ATP-induced cytotoxicity and cell death, cells were treated with *S. nigrum* (30 µM) for the same durations, both in the presence and absence of ATP, with Triton X-100 (1%) serving as a positive control for maximum LDH release. Maximum LDH release was defined as the amount of LDH released by cells treated solely with ATP (100 µM), *S. nigrum*, and *S. nigrum* + ATP. Results demonstrated that *S.*

nigrum (30 µM) significantly attenuated ATP-induced cytotoxicity, leading to reduced cell death. Specifically, LDH release increased by approximately 60% in cells treated with ATP (100 µM) compared to controls, whereas treatment with *S. nigrum* and *S. nigrum* + ATP reduced LDH release to levels lower than ATP-treated cells but higher than untreated controls. Importantly, treatment with *S. nigrum* (30 µM) alongside ATP (100 µM) preserved neuronal viability best after 72 hours, suggesting a protective role against ATP-induced neuronal cell death. These findings align with previous studies demonstrating *S. nigrum*'s neuroprotective effects against motoneuron degeneration in rats (Tehranipour & Mousavi, 2013). Further research is warranted to elucidate the underlying mechanism of *S. nigrum's* neuroprotective function against ATP-induced neurotoxicity (Fig. 4).

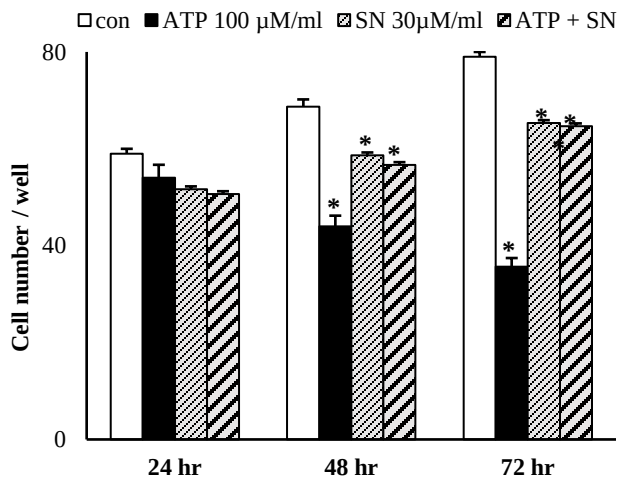

Fig. 1. Effect of *S. nigrum* on proliferation of PC12 cells. PC12 cells were treated with ATP (100M), *S. nigrum (30 µM)* and *S. nigrum (30 µM)* +ATP (100 µM) at indicated doses for 24, 48 and 72 hrs and harvested for determination of cell number by using a trypan blue dye assay. * p<0.001 compared with untreated control.

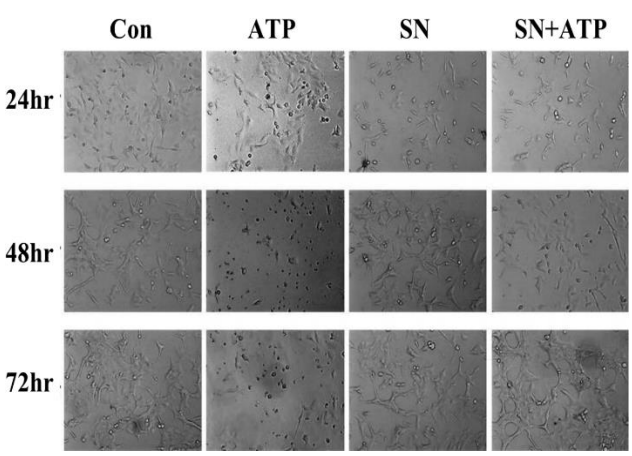

Fig. 2. PC12 cells were cultured in DMEM medium with each reagent (con: vehicle, ATP 100 µM/ml, *S. nigrum* 30 µM/ml, treatment for. 24, 48 and 72 hours and morphological changes were observed thereafter. With ATP 100 µM/ml treatment (first row) cells become shrinking and on a flattened shape. The proliferation looks arrested and cell death progressed after 48 and 72 hours of treatment. In *S. nigrum* treatment, most of the cells healthy and morphology was properly polygonal in shape even in presence of ATP.

Solanum nigrum (SN) has garnered attention for its multifaceted pharmacological properties, ranging from traditional anti-tumor uses to emerging roles in mitigating complex diseases like alcoholic liver disease (ALD). Historically valued in traditional Chinese medicine for its anti-tumor effects (Li *et al*., 2021), SN is now also studied for its antioxidant and anti-inflammatory properties, particularly in the context of SN berry extract (SNE) and ALD (Wang *et al*., 2024). Concurrently, investigations into SN's protective mechanisms against ATP-induced cytotoxicity in PC12 cells reveal its potential in neuroprotection and cellular apoptosis modulation. These studies underscore SN's diverse therapeutic potential, suggesting pathways for further exploration into its applications across different disease models, from cancer treatment to neurodegenerative conditions and liver health management.

ATP, as an extracellular key signaling molecule in the CNS, is increasingly recognized for its role in various pathological and physiological events. Studies indicate that ATP-induced cell death results from reactive oxygen species (ROS) insults triggered by Ca2+-induced oxidative stress, leading to acute apoptosis (Ref). Mitochondrial ROS generation is closely associated with ATP-induced cell death, processes that can be modulated by ATP and influenced by antioxidant interventions (Sun & Chen, 1998). *S. nigrum* has demonstrated neuroprotective properties against motoneuron degeneration in rats (Tehranipour & Mousavi, 2013), attributed in part to its polyphenols' ability to scavenge ROS and chelate transition metal ions. The neuroinflammatory response associated with stroke-related neuronal injury and neurodegenerative diseases has spurred investigations into *S. nigrum*'s antiseizure and antioxidant capabilities (Lin *et al*., 2008; Wannang *et al*., 2008; Bontempo *et al*., 2013) and *Solanum nigrum* have therapeutic effect in protection of organs in rotenone induced Parkinson rat model (Iftikar *et al*., 2024).

Thus, while much remains to be explored regarding the potential effects of *S. nigrum's* glycoalkaloids, this study aimed to investigate its protective properties against ATP-induced cellular damage and programmed cell death in PC12 cells. The findings suggest that pretreatment with *S. nigrum* inhibits LDH release and mitigates ATP toxicity, promoting cell proliferation in a time-dependent manner. This underscores *S. nigrum's* potential in preventing ATP-induced cytotoxicity. Extracellular ATP activates inositol phosphates and mobilizes intracellular calcium, initiating ROS production and apoptosis. Exposure of PC12 cells to ATP increased LDH release and apoptotic cell count, findings corroborated by Hoechst staining revealing morphological changes indicative of apoptosis. Additionally, this study confirms *S. nigrum* 's ability to suppress ATP-induced apoptosis in PC12 cells, while also inducing apoptosis in certain cancer cells (Son *et al*., 2003). Calcium stress, implicated in various neurodegenerative conditions such as Alzheimer's disease, excitotoxicity, and ischemia, has been linked to CPP32 activation and subsequent apoptotic cell death in PC12 cells (Takadera *et al*., 1990).

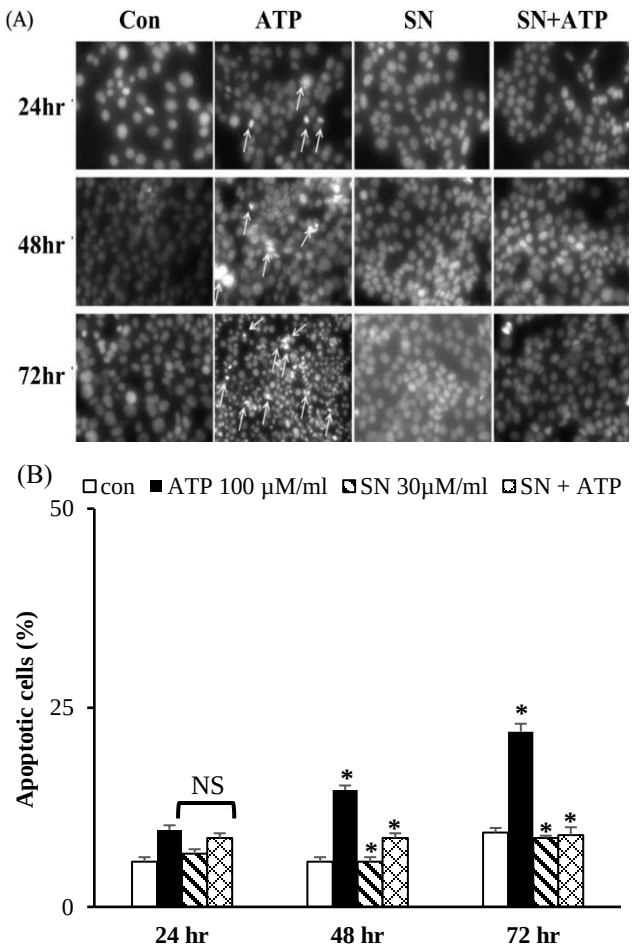

Fig. 3. (A & B) Protective effect of *S. nigrum* (30 µM) on ATP -induced cell damage in the PC12 cell line in terms of reduced apoptosis. The samples were treated for 24, 48 and 72 hours with ATP (100 µM), *S. nigrum* (30 µM) and *S. nigrum* +ATP. Cellular morphological changes were observed under a fluorescence microscope after Hoechst 33258 and % of apoptotic cells were calculated.

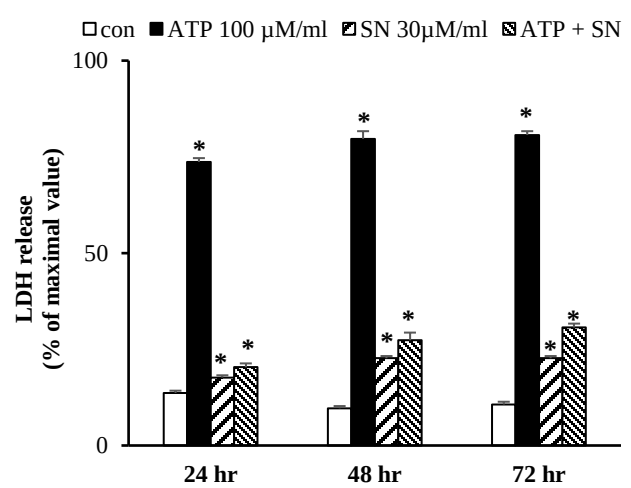

Fig. 4. Release of lactate dehydrogenase (LDH) from damaged neuronal cells after ATP treatment, *S. nigrum*, *S. nigrum* + ATP. LDH activities were measured spectrophotometrically by monitoring the changes of absorbance at 490 nm. Controls include vehicle and for maximum LDH release Triton X-100 (1%). Means are significantly different (p<0.05).

Conclusion

To our knowledge, *S. nigrum* has demonstrated the ability to provide protection against ATP-induced anti-proliferative effects and apoptotic cell death in PC12 cells. This protection may be attributed to the inhibition of apoptosis and antioxidant recovery by *S. nigrum*. However, further research is necessary to determine the involvement of intrinsic and extrinsic pathways in the inhibition of apoptosis by *S. nigrum*. It has been recognized for its neuroprotective properties and has shown potential as a treatment for neurodegenerative conditions like Parkinson's and Alzheimer's diseases. Additionally, more studies are required to investigate whether apoptosis and oxidative stress are linked to calcium signaling and pathways.

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