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Evaluating the Biological Potential of Alternanthera Sessilis Methanolic Extract: Antioxidant, Anti-Inflammatory, and Neurodegenerative Disease Management

Jyothirmayee Devineni[1*], Karunasree Varicola[2], Shaik Karishma Kowsar[3], Priyanka Devarakonda[4], HimaUdayasree.G[5], Alluru Bhavana[6], Rekapalli Neelima Devi[7]

[1]Department of Pharmaceutics and Biotechnology, KVSR Siddhartha College of Pharmaceutical Sciences, Vijayawada, Andhra Pradesh, India. Pin-520010.
[2]Department of Pharmacognosy, KVSR Siddhartha College of Pharmaceutical Sciences, Vijayawada, Andhra Pradesh, India. Pin-520010.
[3,4,5,6,7] Research Scholar, Department of Pharmaceutics and Biotechnology, KVSR Siddhartha College of Pharmaceutical Sciences, Vijayawada, Andhra Pradesh, India, Pin-520010.

Corresponding Author
Jyothirmayee Devineni[1*]
Department of Pharmaceutics and Biotechnology, KVSR Siddhartha College of Pharmaceutical Sciences, Vijayawada, Andhra Pradesh, India. Pin-520010.

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ABSTRACT:

The study investigates the therapeutic potential of the methanolic extract of Alternanthera sessilis (ASLE-M) through extensive phytochemical screening and various biological assays. ASLE-M was found to contain a wide array of bioactive compounds, including alkaloids, saponins, sterols, phenols, carbohydrates, tannins, flavonoids, fatty acids, terpenoids, and glycosides, indicating its diverse pharmacological properties. The reducing power assay demonstrated strong antioxidant activity, with ASLE-M effectively scavenging reactive oxygen species (ROS) and mitigating oxidative stress. Cytokine profiling revealed significant inhibition of key pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) in RAW264.7 cells, highlighting ASLE-M's potent anti-inflammatory effects. Additionally, ASLE-M exhibited notable neuroprotective properties by reducing ROS levels in SK-N-SH neuronal cells, thereby enhancing cellular antioxidant defenses and supporting neuronal health. These findings suggest that ASLE-M could be a valuable source of natural compounds for developing treatments against oxidative stress, inflammation, and neurodegenerative diseases. The study underscores the need for further in vivo studies and clinical trials to fully elucidate the therapeutic mechanisms and validate the efficacy of ASLE-M in various medical applications, particularly in managing inflammatory conditions and neurodegenerative disorders.

Keywords: Neuroprotective, RAW264.7 Cells, Lipoxygenase, Antioxidant, SK-N-SH Cells, Alternanthera Sessilis

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1. Introduction

Oxidative stress is a condition characterized by an imbalance between the production of reactive oxygen species (ROS) and the body's ability to neutralize them with antioxidants. ROS, which include free radicals like superoxide and non-radical species such as hydrogen peroxide, are natural byproducts of normal cellular metabolism. Under physiological conditions, ROS play essential roles in cell signalling, homeostasis, and defense against pathogens. However, when produced in excess, ROS can cause significant cellular damage by oxidizing lipids, proteins, and DNA. This oxidative damage disrupts cellular function and can trigger cell death pathways. Antioxidants, both endogenous and exogenous, act to neutralize ROS and mitigate their harmful effects. Endogenous antioxidants include enzymes like superoxide dismutase, catalase, and glutathione peroxidase, while exogenous antioxidants can be obtained from the diet, such as vitamins C and E. The delicate balance between ROS production and antioxidant defenses is crucial for maintaining cellular health and function (Miraj and Kiani, 2016, Jakovljević et al., 2019, Eidi et al., 2005).
While they play essential roles in cell signalling and homeostasis, excessive ROS can cause significant cellular damage. This damage occurs through the oxidation of DNA, proteins, and lipids, leading to impaired cellular function and death. The brain, due to its high oxygen consumption and abundant lipid content, is particularly vulnerable to oxidative stress (Shoulson and Parkinson Study, 1998, Jain, 2011). Neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (ALS), are characterized by the progressive loss of structure or function of neurons, leading to their death. These diseases are strongly associated with oxidative stress. For example, in Alzheimer's disease, oxidative damage is believed to contribute to the formation of amyloid-beta plaques and tau tangles, which are hallmark features of the disease. Similarly, in Parkinson's disease, oxidative stress is linked to the degeneration of dopaminergic neurons in the substantia nigra, a brain region crucial for movement control (Jain, 2011, Shabab et al., 2017, DiSabato et al., 2016). The connection between oxidative stress and neurodegeneration underscores the potential for neuroprotective strategies that mitigate oxidative damage. Neuroprotection refers to the mechanisms and strategies used to protect neuronal structure and function from damage. These strategies can be pharmacological, involving the use of drugs and compounds that possess antioxidant properties, or they can be lifestyle-based, such as diet and exercise interventions that enhance the body's natural antioxidant defences (DiSabato et al., 2016, Ghorbani and Esmaeilizadeh, 2017).
Several compounds have been investigated for their neuroprotective potential through the reduction of oxidative stress. Antioxidants like vitamin E, vitamin C, and glutathione are known to scavenge ROS directly, thereby preventing cellular damage. Other compounds, such as flavonoids found in fruits and vegetables, have been shown to enhance the body's endogenous antioxidant systems. For instance, resveratrol, a polyphenol found in grapes, has demonstrated neuroprotective effects in experimental models of neurodegenerative diseases by activating antioxidant pathways and reducing oxidative stress (DiSabato et al., 2016, Lyman et al., 2014).
Herbal extracts and natural products are also gaining attention for their potential neuroprotective effects. For example, Ginkgo biloba extract has been studied for its ability to improve cognitive function and reduce oxidative damage in the brain. Similarly, curcumin, the active component of turmeric, has shown promise in preclinical studies for its antioxidant and anti-inflammatory properties, making it a candidate for neuroprotection (Zouhri et al., 2023, Pinto et al., 2024, Sarma Kataki et al., 2012). In addition to direct antioxidant effects, some neuroprotective strategies aim to enhance the brain's resilience to oxidative stress. This can involve upregulating endogenous antioxidant enzymes such as superoxide dismutase (SOD),

catalase, and glutathione peroxidase. Compounds that activate transcription factors like Nrf2, which regulates the expression of antioxidant proteins, are being explored for their potential to boost the brain's defense mechanisms (Lyman et al., 2014, Kataki et al., 2014). The development of effective neuroprotective therapies is crucial given the rising prevalence of neurodegenerative diseases and the current lack of curative treatments. While many compounds show promise in preclinical studies, translating these findings to clinical practice remains challenging. Factors such as bioavailability, blood-brain barrier penetration, and long-term safety need to be thoroughly evaluated (Landskron et al., 2014, Zhang and An, 2007).
In assumption, oxidative stress plays a significant role in the pathogenesis of neurodegenerative diseases, making it a critical target for neuroprotective strategies. By reducing oxidative damage and enhancing the brain's antioxidant defences (Kataki et al., 2014, Zouhri et al., 2023, Pinto et al., 2024), it may be possible to slow the progression of neurodegenerative diseases and improve the quality of life for affected individuals. Ongoing research into antioxidants, natural products, and pharmacological agents continues to advance our understanding of neuroprotection and holds promise for the development of effective therapies (Choi et al., 2023, Hamad et al., 2023, Mayangsari et al., 2023).

Alternanthera sessilis, commonly known as sessile joyweed or dwarf copperleaf, is a perennial herbaceous plant belonging to the Amaranthaceae family (Walter et al., 2014). It is widely distributed across tropical and subtropical regions, including parts of Asia, Africa, and the Americas. This hardy plant is known for its adaptability to various environmental conditions, thriving in wet, marshy areas as well as dry, sandy soils. The leaves of Alternanthera sessilis are small, elliptic, and often display a vibrant green or purplish hue, making it a popular choice for ornamental gardening and aquariums (Walter et al., 2014, Hwong et al., 2022). Traditionally, Alternanthera sessilis has been utilized in various cultural practices and folk medicine. In Ayurvedic and traditional Chinese medicine, it is valued for its wide range of therapeutic properties. The plant is reputed for its anti-inflammatory, antioxidant, antimicrobial, and anti-diabetic activities. It is often used to treat ailments such as fever, wounds, digestive disorders, and respiratory conditions (Hwong et al., 2022, Shehzad et al., 2018). Recent scientific studies have begun to validate these traditional uses, highlighting the potential pharmacological benefits of Alternanthera sessilis. Research has focused on its rich phytochemical composition, which includes flavonoids, alkaloids, saponins, tannins, and phenolic compounds. These bioactive constituents contribute to its medicinal properties and offer promising avenues for the development of new therapeutic agents (Hwong et al., 2022, Shehzad et al., 2018). In light of these facts, the current study aimed to evaluate the neuroprotective and antioxidative qualities of cold-macerated methanolic leaf extract Alternanthera sessilis.

2. Material and Methods

Plant collection and authentication

Between October and November of 2023, a botanist gathered Alternanthera sessilis in the Kullu region of Himachal Pradesh, India. This specimen was kept in the herbarium of our lab and was labelled MK-2023-312. We obtained lipopolysaccharide (LPS) and NG-monomethyl-l-arginine (N-MMA) from Sigma Aldrich, Mumbai, India. Foetal bovine serum and RPMI1640 were purchased from Himedia in Mumbai, India. From ATCC (Rockville, MD), the RAW264.7 and SK-N-SH cell lines were acquired. The remaining compounds were all analytical grade, and they were bought from Sigma Aldrich and Loba Chem in India.

Extraction of the plant

To extract the compounds from Alternanthera sessilis leaves, 100 g of air-dried leaves were ground into a fine powder. The powder was then subjected to cold maceration using 92% methanol (2×2 Liters) at 25°C. After the extraction process, the mixture was filtered, and the filtrate was concentrated under vacuum and heated to dryness under reduced pressure. The resulting methanolic extract was labelled ASLE-M.

Preliminary Phytochemical screening

The methanolic extract of Alternanthera sessilis (ASLE-M) underwent a comprehensive preliminary phytochemical screening to identify the presence of various classes of phytocompounds. Standard qualitative tests were employed based on the methodologies outlined elsewhere (Shaikh and Patil, 2020, Raaman, 2006). To detect alkaloids, Mayer's, Wagner's, and Dragendorff's reagents were used, which result in the formation of precipitates if alkaloids are present. Flavonoids were identified using the alkaline reagent test and lead acetate test, where a colour change indicates a positive result. Tannins and phenolic compounds were detected using the ferric chloride test and gelatin test, which produce a blue-black or green precipitate, respectively. Saponins were identified through the froth test and foam test, which involved observing the formation of stable froth. Glycosides were detected using the Keller-Kiliani test and Borntrager's test, which are characterized by specific colour changes indicating the presence of cardiac glycosides. Terpenoids and steroids were confirmed through the Salkowski test and Liebermann-Burchard test, which develop characteristic colours. Carbohydrates were detected using Molisch's test and Benedict's test, where colour changes or precipitate formation indicate their presence. Proteins and free amino acids were identified using the Biuret test and Ninhydrin test, respectively, both of which result in specific colour reactions. Lastly, fats and oils were detected using the Sudan III stain test, which stains lipids. Each of these tests provided qualitative insights into the phytochemical composition of the ASLE-M extract, laying the groundwork for further studies on its potential bioactive properties.

Cell culture

The cultivation of SK-N-SH and RAW264.7 cell lines was carried out using RPMI1640 medium, which was meticulously prepared to support cell growth. The medium was supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin to prevent bacterial contamination. Additionally, 10% fetal bovine serum (FBS) was added to provide essential nutrients, growth factors, and hormones necessary for cell proliferation and survival. The cells were maintained in a controlled environment to ensure optimal growth conditions. They were incubated at a temperature of 37°C, which is ideal for the growth of mammalian cells. The incubator was set to maintain a humidified atmosphere with 5% carbon dioxide (CO_2). This CO_2 concentration is crucial for maintaining the pH of the culture medium, as it helps to balance the bicarbonate buffer system within the medium. Regular monitoring and maintenance of the cell cultures were performed to ensure their health and viability. The cells were observed under a microscope to check for confluency, morphology, and any signs of contamination. Medium changes were conducted every 2-3 days to provide fresh nutrients and remove metabolic waste products. Sub-culturing or passaging of the cells was done when they reached around 80-90% confluency to prevent overcrowding and ensure continuous growth. This rigorous cell culture protocol ensured that the SK-N-SH and RAW264.7 cell lines were kept in optimal conditions, providing a reliable and consistent platform for subsequent experimental procedures. The carefully controlled environment and detailed maintenance routines are essential for obtaining accurate and reproducible results in cell-based research.

Antioxidant activity

Reducing power assay: To determine the reducing power of the plant extract, we prepared various concentrations of the extract in distilled water, typically ranging from 25 to 100 µg/ml. For comparison, standard solutions of ascorbic acid were prepared at the same concentrations. This setup allowed us to assess the reducing power of the plant extract relative to a known antioxidant. The assay began by mixing 2.5 ml of phosphate buffer (0.2 M, pH 6.6) with 2.5 ml of potassium ferricyanide (1%) in test tubes. To these mixtures, 1 ml of each concentration of the plant extract was added. In parallel, 1 ml of each concentration of ascorbic acid was added to separate mixtures of phosphate buffer and potassium ferricyanide. The test tubes were then incubated at 50°C for 20 minutes in a water bath to facilitate the reduction reaction. After incubation, the reaction was halted by adding 2.5 ml of trichloroacetic acid (10%) to each test tube, ensuring thorough mixing. The mixtures were subsequently centrifuged at 3000 rpm for 10 minutes to separate the precipitate. Following centrifugation, 2.5 ml of the supernatant was carefully collected from each test tube and transferred to new tubes. To develop the colour necessary for spectrophotometric analysis, 2.5 ml of distilled water and 0.5 ml of ferric chloride (0.1%) were added to each supernatant sample. The resulting solutions were mixed well and their absorbance was measured at 700 nm using a spectrophotometer. This step was crucial for quantifying the reducing power of the plant extract. The absorbance values obtained were plotted against the concentrations of both the plant extract and the ascorbic acid standard. An increase in absorbance with increasing concentration indicated a stronger reducing power. By comparing the absorbance values of the plant extract with those of the ascorbic acid standard, we could evaluate the antioxidant potential of the extract. This method provided a reliable assessment of the reducing power of the plant extract. Higher absorbance values indicated a greater capacity to reduce Fe^{3+} to Fe^{2+}, reflecting stronger antioxidant activity. The assay was conducted in triplicate to ensure accuracy and reproducibility, with careful attention given to the cleanliness of glassware and reagents to avoid contamination and errors.

Neuroprotective activity

Cell culture and treatment: To investigate the neuroprotective effects of WS, SK-N-SH cells, a human neuroblastoma cell line obtained from the American Type Culture Collection (ATCC), were cultured using DMEM (Dulbecco's Modified Eagle Medium). The medium was supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin to provide the necessary nutrients and antibiotics for cell growth and maintenance. The cells were seeded into 96-well plates (Corning) at a density of 1.5×10^4 cells per well. Following seeding, the cells were incubated at 37°C in an atmosphere containing 5% CO_2 and humidified air, which ensured optimal growth conditions. After 24 hours, the medium was completely withdrawn to synchronize the cells and minimize variations in nutrient availability. Subsequently, the cells were maintained in DMEM containing antibiotics but without serum to induce a state of nutrient deprivation, simulating a stress condition that might enhance the detection of WS's neuroprotective effects. Following this serum deprivation, the cells were exposed to acrolein at a concentration of 20 µM. Acrolein, a potent and toxic aldehyde, was used to induce oxidative stress and cellular damage, mimicking conditions of neurodegenerative diseases. The cells were treated with acrolein for a full day (24 hours) to allow sufficient time for the development of oxidative stress and subsequent cellular responses. This treatment setup aimed to provide a robust model for assessing the potential neuroprotective properties of WS by evaluating the cells' viability, morphological changes, and biochemical markers of oxidative stress after exposure to the toxic agent. This experimental approach ensures a controlled environment for testing the neuroprotective effects of ASLE-M, providing reliable and reproducible data on its efficacy in mitigating acrolein-induced neurotoxicity. The inclusion of appropriate controls and replicates is crucial to validate the

findings and establish the therapeutic potential of ASLE-M in neuroprotection (Thummayot et al., 2016, Thummayot et al., 2014).

Intracellular reactive oxygen species level

To verify the antioxidant activity of the methanolic extract of Alternanthera sessilis (ASLE-M), we evaluated its capacity to scavenge reactive oxygen species (ROS) in SK-N-SH cells using the fluorescent dye 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA). This method, based on previously established protocols, provides a reliable measure of intracellular ROS levels. The procedure began by incubating SK-N-SH cells with the DCF-DA dye, which is cell-permeable and non-fluorescent in its native state. Once inside the cell, DCF-DA is enzymatically cleaved by intracellular esterases to 2',7'-dichlorodihydrofluorescein (DCFH), a non-fluorescent compound. DCFH is then rapidly oxidized by ROS present within the cells to form 2',7'-dichlorofluorescein (DCF), a highly fluorescent molecule. The fluorescence intensity of DCF is directly proportional to the amount of ROS within the cells. Therefore, by measuring the fluorescence intensity using a fluorescence microplate reader or fluorescence microscope, we can quantitatively assess the intracellular ROS levels. Higher fluorescence intensity indicates higher ROS levels, whereas lower fluorescence intensity suggests effective ROS scavenging by the antioxidant compound being tested. In our study, SK-N-SH cells were treated with varying concentrations of ASLE-M to determine its ROS scavenging capacity. After treatment, the cells were incubated with DCF-DA dye under conditions conducive to the dye's conversion and oxidation. The fluorescence intensity of the resulting DCF was then measured, providing a direct correlation between the presence of intracellular ROS and the efficacy of ASLE-M as an antioxidant. By comparing the fluorescence intensity of ASLE-M treated cells with untreated control cells, we were able to determine the extent to which ASLE-M reduced intracellular ROS levels. This comparative analysis confirmed the antioxidant potential of ASLE-M, highlighting its capacity to neutralize ROS and protect cells from oxidative stress. This method provides a robust and sensitive approach to evaluating the antioxidant activity of compounds within a cellular context, offering valuable insights into their potential therapeutic applications in mitigating oxidative stress-related damage (Thummayot et al., 2014, Ramassamy and Singh, 2017).

Cytokines profiling (IL-1β, IL-6 and TNF-α)

The cytokine profiling indicating the effect of Alternanthera sessilis (ASLE-M) extract was performed to evaluate its protective properties by measuring its effects on the production of key pro-inflammatory cytokines: interleukin-1 beta (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α). For this analysis, ASLE-M extract was prepared at a concentration of 100 mg/ml by solubilizing it in a solution consisting of 50% ethanol and 50% phosphate-buffered saline (PBS). This solution was then diluted with RPMI1640 medium prior to treatment. To assess the inhibitory effects of the ASLE-M extract on cytokine production, RAW264.7 macrophage cells were treated with lipopolysaccharide (LPS) to induce an inflammatory response. Each fraction of the extract was tested at a concentration of 100 µg/ml. Following the treatment, the culture supernatants were collected for cytokine analysis. The supernatants were subjected to enzyme-linked immunosorbent assay (ELISA) to quantify the levels of IL-1β, IL-6, and TNF-α. ELISA is a highly sensitive and specific technique that uses antibodies to detect and measure the concentration of cytokines in the samples. By comparing the cytokine levels in LPS-treated cells with and without the ASLE-M extract, the study aimed to determine the extract's potential to reduce inflammation. The results of this analysis provided insight into the protective activity of ASLE-M, showing its ability to modulate cytokine production in response to an inflammatory stimulus. Reduced levels of IL-1β, IL-6, and TNF-α in the treated samples indicated that ASLE-M could effectively inhibit the production of these

pro-inflammatory cytokines, suggesting its potential as a therapeutic agent for inflammatory conditions (Lee et al., 2006).

Statistical Analysis

For all in vitro experiments conducted in this study, the data were presented as the mean ± standard deviation (SD). Each experiment was performed in triplicate to ensure the reliability and reproducibility of the results. The statistical analysis was conducted using GraphPad Prism software. To compare the results of neuroprotective and antioxidant tests, a one-way analysis of variance (ANOVA) was employed. The significance threshold was set at $p < 0.05$ (2-tailed), indicating that results with a p-value less than 0.05 were considered statistically significant. Turkey's multiple comparison test as Post hoc test were applied following the ANOVA to pinpoint specific differences between groups.

3. Results

Preliminary Phytochemical screening

The preliminary phytochemical screening of Alternanthera sessilis leaf methanolic extract (ASLE-M) revealed the presence of a broad spectrum of bioactive compounds. The extract tested positive for alkaloids, saponins, sterols, phenols, carbohydrates, tannins, flavonoids, fatty acids, terpenoids, and glycosides. This diverse phytochemical profile suggests that ASLE-M has significant therapeutic potential due to the wide range of biological activities associated with these compounds. Alkaloids and flavonoids are known for their antioxidant and anti-inflammatory properties, while saponins and tannins exhibit antimicrobial activities. Sterols and terpenoids contribute to anti-inflammatory and anticancer effects. The presence of phenols and glycosides further enhances the extract's potential for antioxidant activity. The comprehensive presence of these phytoconstituents indicates that ASLE-M could be a valuable source of natural compounds for developing treatments against various diseases, particularly those involving oxidative stress and inflammation.

Table 1. Results of Preliminary Phytochemical screening

Phytoconstituents	**ASLE-M**
Alkaloids	√
Saponins	√
Sterols	√
Phenols	√
Carbohydrates	√
Tannins	√
Flavanoids	√
Fatty acid	√
Terpenoid	√
Glycosides	√

Antioxidant activity

Reducing power assay: The figure 1 presents the results of a reducing power assay, showing the absorbance at 700 nm as a function of concentration for three different samples: ASLE-M, Butylated Hydroxyanisole (BHA), and α-Tocopherol. In a reducing power assay, higher absorbance indicates greater electron-donating ability, which correlates with stronger antioxidant activity. The ASLE-M extract demonstrates a significant increase in absorbance with rising concentration, indicating a strong reducing power. At the highest concentration (270

μg/ml), ASLE-M shows the highest absorbance among the three samples, suggesting superior antioxidant activity compared to BHA and α-Tocopherol. BHA shows a consistent increase in absorbance with increasing concentration, reflecting its known antioxidant properties. Although effective, BHA's reducing power is lower than that of ASLE-M at higher concentrations, suggesting that ASLE-M might be a more potent antioxidant. α-Tocopherol exhibits the lowest absorbance values across all concentrations, indicating the least reducing power among the three samples. Despite its known benefits as an antioxidant, α-Tocopherol's performance in this assay is less impressive, which may be due to the specific conditions and nature of the reducing power assay. The reducing power assay results indicated that ASLE-M has a significant electron-donating capability, surpassing both BHA and α-Tocopherol at higher concentrations. This suggests that ASLE-M possesses strong antioxidant properties, making it a promising candidate for natural antioxidant applications. The high reducing power of ASLE-M highlights its potential utility in mitigating oxidative stress and protecting against oxidative damage in biological systems.

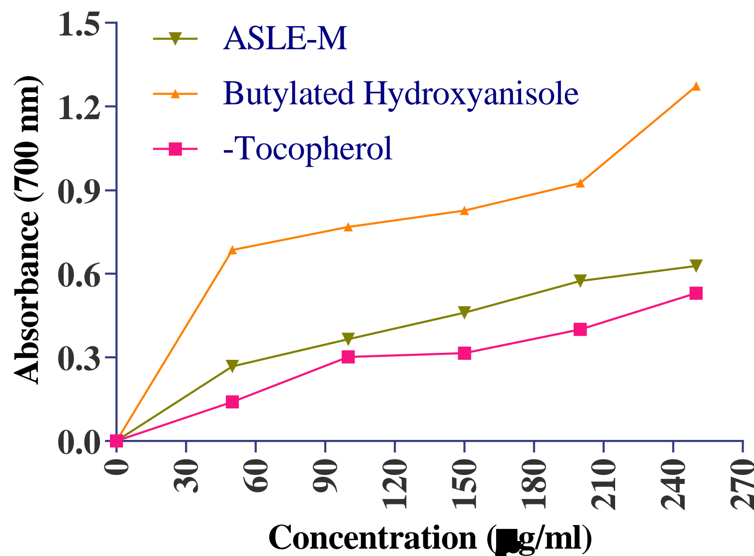

Figure 1. Reducing power assay of ASLE-M

Neuroprotective activity

ASLE-M decreases intracellular reactive oxygen species level: The table 2 and figure 2 illustrates the effect of varying concentrations of ASLE-M (Alternanthera sessilis leaf methanolic extract) on the levels of intracellular reactive oxygen species (ROS) in a controlled experiment. The ROS levels are indicated by absorbance at 700 nm, with higher values representing greater oxidative stress. The baseline ROS level for untreated cells, set as the control, is 100 (mean ± SD), representing normal intracellular ROS levels without any induced oxidative stress. When 500 μM hydrogen peroxide (H2O2) was introduced, there was a significant increase in ROS levels to 292, indicating a substantial oxidative stress environment. At a concentration of 3 μg/ml, ASLE-M showed minimal protective effect against oxidative stress, with ROS levels remaining almost as high as with H2O2 alone. This suggests that at this lower concentration, ASLE-M does not have a significant impact on reducing ROS. However, at 6 μg/ml, a noticeable reduction in ROS levels to 251 was observed, indicating some antioxidant activity of ASLE-M. This trend continued as the concentration increased, with ROS levels dropping to 233 at 12 μg/ml, showing increased antioxidant protection. Significant reductions in ROS levels were observed at higher concentrations of ASLE-M. At 24 μg/ml,

ROS levels decreased to 173, demonstrating strong antioxidant properties. The lowest ROS levels were recorded at 48 µg/ml, with an absorbance of 169, indicating the highest antioxidant activity at this concentration. These findings suggest that ASLE-M exhibits a dose-dependent antioxidant effect, with higher concentrations providing greater protection against oxidative stress. The data clearly show that ASLE-M is effective in mitigating oxidative stress in a dose-dependent manner. Higher concentrations of ASLE-M (24 and 48 µg/ml) significantly reduce intracellular ROS levels, demonstrating its potential as a natural antioxidant. This indicates that ASLE-M could be a promising candidate for protecting cells from oxidative damage induced by agents like hydrogen peroxide. Further studies are needed to explore the exact mechanisms and bioactive compounds responsible for these protective effects, which could contribute to the development of new therapeutic strategies for managing oxidative stress-related conditions.

Table 2. Impact of ASLE-M in intracellular reactive oxygen species level

Control	**H_2O_2**	**ASLE-M Concentration (µg/ml)**				
	500 µm	**3**	**6**	**12**	**24**	**48**
100	292±6.07	291±5.17	251±5.99	233±4.18	173±2.25	169±2.58

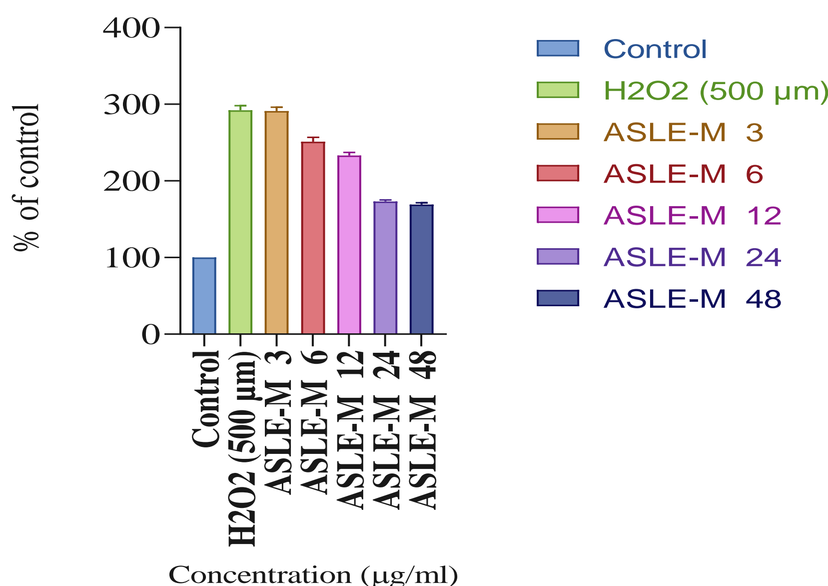

Figure 2. ASLE-M decreases intracellular reactive oxygen species level.

Cytokines profiling (IL-1β, IL-6 and TNF-α)

The table 3 presents the inhibitory effects of 100 mg/ml Alternanthera sessilis leaf methanolic extract (ASLE-M) on the production of key pro-inflammatory cytokines - IL-6, IL-1β, and TNF-α—in RAW264.7 cells. The percentages represent the degree of inhibition of cytokine production compared to untreated control cells, with lower values indicating greater inhibition.

IL-6 Inhibition: ASLE-M exhibited a significant inhibition of IL-6 production, with a mean inhibition percentage of 57.97 ± 1.13. This suggests a strong anti-inflammatory effect, as IL-6 is a major cytokine involved in the inflammatory response. The high level of inhibition indicates that ASLE-M effectively suppresses the synthesis or release of IL-6 in activated macrophages, reducing the overall inflammatory response.

IL-1β Inhibition: For IL-1β, ASLE-M showed an inhibition percentage of 43.86 ± 1.23, also significant at $p < 0.01$. IL-1β is another critical pro-inflammatory cytokine that plays a role in the regulation of immune and inflammatory responses. The ability of ASLE-M to significantly

inhibit IL-1β production further highlights its potential as an anti-inflammatory agent, capable of modulating key pathways involved in inflammation.

TNF-α Inhibition: TNF-α production was inhibited by 49.89 ± 1.09% in the presence of ASLE-M. TNF-α is a pivotal cytokine in inflammation and is involved in the pathogenesis of various inflammatory and autoimmune diseases. The substantial inhibition of TNF-α production by ASLE-M indicates its strong anti-inflammatory potential and supports its use in conditions characterized by excessive inflammation.

The data demonstrate that ASLE-M significantly inhibits the production of IL-6, IL-1β, and TNF-α in RAW264.7 cells, with inhibition percentages of 57.97%, 43.86%, and 49.89%, respectively. These results indicate that ASLE-M possesses potent anti-inflammatory properties, capable of downregulating key pro-inflammatory cytokines involved in the inflammatory response. The significant p-values (** $p < 0.01$) suggest that the observed effects are highly reliable and not due to random chance. These findings support the potential therapeutic application of ASLE-M in treating inflammatory conditions. By effectively reducing the levels of critical pro-inflammatory cytokines, ASLE-M could help in managing diseases characterized by chronic inflammation, such as arthritis, inflammatory bowel disease, neurodegenerative diseases and other autoimmune disorders. Further research is warranted to elucidate the precise mechanisms by which ASLE-M exerts its anti-inflammatory and protective effects and to explore its efficacy in in vivo models and clinical settings.

Table 3. Impact of 100 mg/ml ASLE-M on pro-inflammatory cytokine production in RAW264.7 cells.

	Inhibition (%)		
Fraction	**IL-6**	**IL-1**	**TNF-α**
ASLE-M	57.97 ± 1.13**	43.86 ± 1.23**	49.89 ± 1.09**

* $p < 0.05$. ** $p < 0.01$

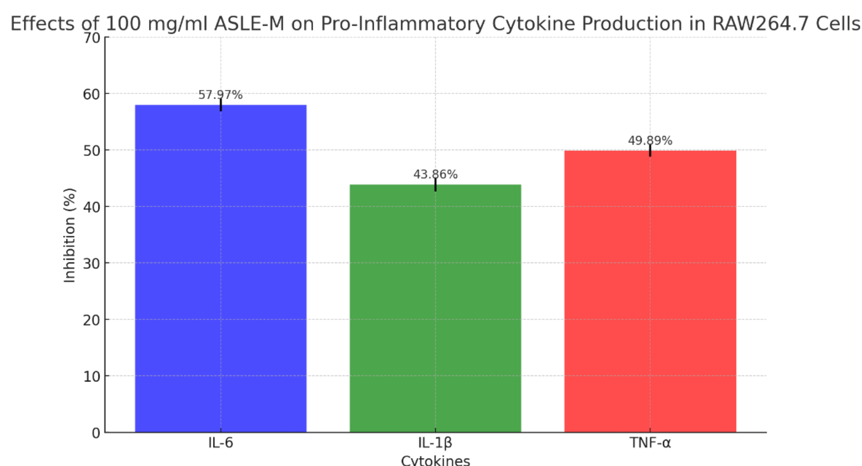

Figure 3. Pro-inflammatory cytokines profiling

4. Conclusion

The study highlighted the significant therapeutic potential of the methanolic leaf extract of Alternanthera sessilis (ASLE-M) through comprehensive phytochemical screening and biological & pharmacological screening and assays. The presence of a broad spectrum of bioactive compounds, including alkaloids, saponins, sterols, phenols, carbohydrates, tannins, flavonoids, fatty acids, terpenoids, and glycosides, underscored the extract's diverse pharmacological properties. These phytoconstituents are known to contribute to various

biological activities such as antioxidant, anti-inflammatory, antimicrobial, and anticancer effects. The reducing power assay demonstrated that ASLE-M exhibits strong antioxidant activity, capable of effectively scavenging reactive oxygen species (ROS) and mitigating oxidative stress. This suggests its potential utility in managing conditions related to oxidative damage. Additionally, the evaluation of ASLE-M's neuroprotective effects showed its potential in protecting neuronal cells from oxidative stress-induced damage. The reduction in ROS levels in SK-N-SH cells treated with ASLE-M highlights its ability to enhance cellular antioxidant defences and support neuronal health. Further, the cytokine profiling revealed that ASLE-M significantly inhibits the production of key pro-inflammatory cytokines (IL-6, IL-1β, and TNF-α) in RAW264.7 cells. This indicates robust anti-inflammatory properties, making ASLE-M a promising candidate for treating inflammatory and autoimmune disorders. The findings supported the use of Alternanthera sessilis as a valuable source of natural antioxidants and anti-inflammatory and neuroprotective agents. Further research, including in vivo studies and clinical trials, is warranted to fully elucidate the therapeutic mechanisms and validate the efficacy of ASLE-M in various medical applications.

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