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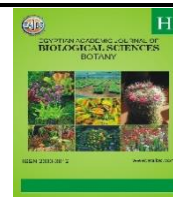
EGYPTIAN ACADEMIC JOURNAL OF BIOLOGICAL SCIENCES BOTANY



ISSN 2090-3812

www.eajbs.com

Vol. 15 No.2 (2024)



Harnessing Spirulina Extract to Enhance Drought Tolerance in Wheat: A Morphological, Molecular Genetic and Molecular Docking Approach

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ARTICLE INFO

Article History

Received:16/11/2024

Accepted:20/12/2024

Available:24/12/2024

Keywords:

Wheat, Drought Stress, Spirulina extract treatment, Gene expression, Molecular docking.

ABSTRACT

Wheat (*Triticum aestivum* L.), a globally important staple crop, faces significant production challenges due to climate change, particularly drought stress. Spirulina is known for its high nutritional value and potential biostimulant properties. Therefore, this study investigated *Spirulina platensis* extract (SpEx) potential to mitigate drought stress effects in wheat, integrating morphological measurements, gene expression data, and molecular docking. SpEx treatment significantly mitigated drought-induced reductions in plant height (18.2%), fresh weight (29.7%), and dry weight (15.7%). Gene expression analysis demonstrated upregulation of stress-responsive genes under SpEx treatment, with peroxidase showing the highest increase (6.13-fold) at 20 mg/ml SpEx concentration, followed by cytochrome P450 (5.23-fold), protein kinase (4.56-fold), and UDPGT (3.67-fold). GC-mass spectrometry analysis of SpEx revealed 27 bioactive compounds. Molecular docking studies of the SpEx-derived compounds with six key proteins (UDPGT, Cytochrome P450, Peroxidase, Protein kinase, ROS, and PCD) revealed varying binding affinities. Four compounds (Araguspongin, Lupeol, Silybin, and Strophanthidine) showed strong interactions with ROS and PCD proteins, exhibiting binding affinities superior to control ligands. ADMET analysis of these four compounds against ROS and PCD proteins demonstrated favorable pharmacological properties, with Araguspongin and Strophanthidine showing high GI absorption and all compounds complying with Lipinski's Rule of Five. Hierarchical cluster analysis of both morphological and gene expression data confirmed distinct grouping patterns between treated and untreated plants. These findings suggest that SpEx enhances wheat drought tolerance through multiple mechanisms, including improved morphological adaptations, enhanced stress-responsive gene expression, and molecular interactions with key stress-related proteins, offering promising applications for sustainable agriculture under water-limited conditions.

INTRODUCTION

Cereal crops are the basis of agricultural production in most countries. *Triticum aestivum* L., as one of the most commonly cultivated cereals in the world (next to rice and maize), is particularly important (Baloglu *et al.*, 2014). Water deficits represent a major global abiotic stress that limit plant productivity by inhibiting plant growth and development.

The consequence of these changes is a reduction in crop quality and quantity, which causes crop yield losses (Zalibekov, 2011, Maqsood *et al.*, 2012 and Hossain, *et al.*, 2013).

Plant responses to drought stress involve complex molecular mechanisms, particularly the regulation of stress-responsive genes. Abiotic stresses can induce irreversible changes in plants, resulting in restricted cell division and growth, reduced fertility, accelerated senescence, and in severe cases, cell death. Protein Kinases (PK) and peroxidase serve as key molecular regulators in plant stress responses. While PKs orchestrate stress sensing and signal transduction pathways, peroxidases contribute to antioxidant defense mechanisms and metabolic signaling. Together, these enzymes form an integral part of the plant's molecular machinery for adapting to various environmental stresses (Zhu, 2016; Qi *et al.*, 2018, Gong *et al.*, 2020).

Cytochrome P450s (CYPs) represent another crucial gene family involved in drought stress responses. As the largest enzyme family catalyzing NADPH- and/or O₂-dependent hydroxylation reactions across all domains of life, CYPs are integral to hormone signaling pathways that regulate plant stress responses (Tamiru *et al.*, 2015, Pinot and Beisson, 2011, Heitz *et al.*, 2012). These enzymes have demonstrated protective functions against various environmental stresses, including drought, heat, salt, and heavy metal exposure (Tamiru *et al.*, 2015, Qin, *et al.*, 2008, Wang *et al.*, 2017 (b), and Rai *et al.*, 2015). Moreover, CYPs are directly involved in plant secondary metabolism, facilitating the detoxification of both xenobiotics and endogenous metabolic byproducts produced under stress conditions.

The plant family UDP-glycosyltransferases (UGTs) gene, the largest glycosyltransferase family in plants, also plays a vital role in drought stress response. These enzymes catalyze the transfer of sugar moieties to diverse small molecules, thereby regulating numerous metabolic processes (Li *et al.*, 2017). UGTs are particularly important in modifying plant hormones and secondary metabolites through the transfer of glucuronic acid from UDP-glucuronic acid to various substrates. The extensive array of UGT genes performs essential functions in the processing and regulating of plant secondary metabolites (Yonekura-Sakakibara *et al.*, 2012). Understanding the expression patterns of these genes in response to drought stress and spirulina treatment could provide valuable insights into the underlying mechanisms of stress tolerance.

Programmed Cell Death (PCD) and the accumulation of Reactive Oxygen Species (ROS) are key processes in plant stress responses. Excessive ROS production during drought stress can lead to oxidative damage and trigger PCD, ultimately affecting plant growth and yield. Molecular docking studies can provide insights into potential interactions between spirulina-derived compounds and proteins involved in PCD and ROS pathways, offering a theoretical basis for observed physiological effects (Petrov *et al.*, 2015; and Mittler *et al.*, 2017).

The utilization of natural biostimulants, such as *Spirulina platensis* extracts, is a significant approach to enhance crop resilience to abiotic stresses. These extracts contain a complex matrix of growth regulators and bioactive compounds that demonstrate considerable efficacy in improving plant stress tolerance and growth under adverse conditions (Zhang *et al.*, 2021). The presence of diverse phytohormones and polysaccharides in seaweed extracts enables the modulation of stress-related gene expression and cellular responses (Khan *et al.*, 2018). While the effects of these biostimulants have been documented, the molecular mechanisms underlying their action in wheat (*Triticum aestivum* L.) require further elucidation.

This study aims to investigate the effects of spirulina application on drought-stressed wheat, focusing on morphological characteristics (shoot height, fresh and dry leaf weight) and the expression of key stress-responsive genes (cytochrome P450, UDPGT, protein kinase, and peroxidase). Additionally, molecular docking analysis will be performed to

explore potential interactions between spirulina compounds and proteins involved in cytochrome P450, UDPGT, protein kinase, peroxidase, PCD, and ROS pathways. We hypothesize that spirulina treatment will mitigate the negative effects of drought stress on wheat by modulating gene expression and improving morphological traits. By combining morphological, gene expression, and molecular docking approaches, we seek to provide a comprehensive understanding of spirulina's effects on wheat plants.

MATERIALS AND METHODS

1- *Spirulina platensis* Extract (SpEx) Preparation and Analysis:

Spirulina platensis powder was obtained from Prof. Dr. Ashraf Bakry, Department of Genetics, Ain Shams University.

The bioactive compound of *S. platensis* powder was extracted by 96% ethanol, the sample to solvent ratio of 1: 5 (w / v), under stirring for 24 h. Then, the sample was filtered using Whatman 42 paper to obtain the macerate. The residue obtained was re-extracted with fresh ethanol for 2 cycles. On the other hand, the macerate was then concentrated using a rotary vacuum evaporator at a temperature of 40 °C. The yield of extracts was expressed as (weight of extract/weight of sample) x100%. (Hardiningtyas *et al.*, 2021).

The active compounds of SpEx were assessed using the Agilent 5977A gas chromatograph mass spectrometry system, which is integrated with the GC Clarus 500 Perkin Elmer system. This system features the AutoSampler [AOC-20i + s] for analyzing various compounds and utilizes bound gas chromatography. The chromatograms produced by this equipment are automatically programmed into the machine.

2- Plant Experiment:

2.1 Plant Material and Growth Conditions:

A pure cultivar of wheat (Sakha 95) was obtained from the Ministry of Agriculture, Field Crop Institute, Agriculture Research Center, Giza, Egypt. A homogenous lot of wheat seeds were selected for uniformity of size, shape, and viability. Before germinating, the seeds were surface sterilized by soaking them for 2 minutes in 70% ethanol followed by 2.5% sodium hypochlorite solution for 5 min, and then washed several times with distilled water (Gao *et al.*, 2018).

The sterilized seeds were presoaked in distilled water for Untreated Control and different concentrations of spirulina extract (SpEx) (10 mg/ml and 20 mg/ml) for 15 hours. Germinated seedlings were transferred to pots (30 cm diameter, 40 cm height) containing a mixture of peat, perlite, and vermiculite (2:1:1 v/v/v). Plants were grown in a controlled environment chamber under the following conditions: temperature $22 \pm 2^\circ\text{C}$, relative humidity $60 \pm 5\%$, photoperiod of 16/8 h (light/dark), and photosynthetic photon flux density of $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool white, fluorescent lamps (Zhang *et al.*, 2020).

2.2 Drought Stress Treatment:

Irrigation was applied to maintain sufficient soil moisture in the drought and control cultivars (soil FC was determined on dry weight basis of irrigated pots after keeping saturated soil for 24 h under free drainage). drought stress was applied to Saka-95, a drought-moderate cultivar after 45 days of sowing (DAS), when the plants had three fully expanded trifoliolate leaves during the vegetative stage, by withholding irrigation for 21 days, with regular irrigation of the control varieties maintained throughout the experiment.

2.3 Spirulina Treatment:

The experiment included both seed pre-treatment and foliar application of Spirulina extract (SpEx). Prior to sowing, seeds were soaked in Spirulina extract except for the control group. Foliar treatments were applied at two developmental stages: two weeks and 45 days after sowing. The experimental groups were treated as follows:

- Untreated control groups (Untreated Control and SpEx Cont) received foliar application of distilled water only.
- Drought stress (DS) group received foliar application of distilled water only under water-limited conditions.
- Treatment groups received foliar application of Spirulina extract at two concentrations:
 - SpEx 10 mg/ml: 10 mg/ml Spirulina extract solution
 - SpEx 20 mg/ml: 10 mg/ml Spirulina extract solution

All foliar applications were performed at the same time points (two weeks and 45 days after sowing) to maintain consistent experimental conditions (Alharbi *et al.*, 2022).

After 21 days, all treatments were 66 days old (heading stage), so flag leaf sampling was carried out to assess the effects of drought stress (DS) and Spirulina extract (SpEx) at the morphological and molecular level (Elnajar *et al.*, 2024). Three different plants' leaves were collected from each pot. All tissues were preserved at 80 °C and promptly frozen in liquid nitrogen.

3- Morphological Measurement:

Morphological measurements were measured 21 days after spirulina treatment and carried out as follows: Plant height (cm): measured from the soil surface to the tip of the longest leaf using a ruler (n = 10 plants per treatment). Leaf fresh weight (g): the third fully expanded leaf from the top was excised, and its fresh weight was immediately measured using an analytical balance (Mettler Toledo, Switzerland) (n = 10 leaves per treatment). Then leaf dry weight (g): the same leaves used for fresh weight measurements were oven-dried at 70°C for 72 hours and then weighed (n = 10 leaves per treatment).

4- Gene Expression Analysis

4.1 RNA Extraction and cDNA Synthesis:

Gene expression analysis: Leaf samples will be collected 21 days after last SpEx treatment. Total RNA will be extracted using TRIzol reagent following the manufacturer's instructions. cDNA will be synthesized using a reverse transcription kit.

4.2 Quantitative Real-Time PCR (qRT-PCR):

Quantitative real-time PCR (qRT-PCR) (Applied Biosystems, Step One Plus Real Time-PCR System) will be performed to analyze the expression of cytochrome P450, UDPGT, protein kinase, and peroxidase genes. The β -actin gene will be used as an internal control. Gene-specific primers were designed using Primer 5.0 software and their sequences were F: 5'AGGAGCACGGTAAGATGTCA3', R: 5'TGGAAGGCAGGGTTGATGAT3' for cytochrome P450, F:5'GCCATCTCGTACAACATCGG3', R:5'GCTTCTGGTCCTTGAA CACC3' for the UDPGT gene, F: 5'GATTCCATGTCCGCCGAAACA3', R: 5'GTCCCAGT CCAGCTTCTTCT3' for protein kinase, F: 5'TCTCCTGTGCAGACATCGTT3', R: 5'GT GTTGAGGCCCTTGATGAC3' for peroxidase gene, and F:5'TGACGTGGATATCAGG AAGG3', R: 5'GCTGAGTGAGGCTAGGATGG3' for β -actin. The program used was 2 min at 95° C, followed by 40 cycles of denaturation for 5 s at 95° C, annealing for 10 s at 58-60° C and extension for 20 s at 72° C. Three replicates of real-time PCR reactions were performed for each sample. Relative gene expression will be calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

5- Molecular Docking Analysis:

The molecular docking study was performed to investigate the binding interactions between Spirulina-derived compounds and key proteins involved in programmed cell death (PCD) and reactive oxygen species (ROS) pathways.

5.1 Ligand Preparation:

Active compounds from Spirulina were identified through GC-MS analysis of the ethanolic extract. The 3D structures of these compounds were downloaded from PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) in 3D.sdf format. The compounds were imported into PyRx software as a small library, where energy minimization was performed to optimize

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their conformations. Final ligand preparations were conducted using AutoDock Tools to ensure optimal docking conditions.

5.2 Protein Preparation:

The 3D structures of target proteins involved in UDP-glucuronosyltransferase (UDPGT) (PDB: 7ES0), Protein kinase (PDB: 6TE2), Peroxidase (PDB: 8OGI), and Cytochrome P450 (PDB: 2IJ5), Programmed Cell Death (PCD) (PDB: 4HWH) and Reactive Oxygen Species (ROS) (PDB: 7Z5X) pathways were obtained from the Protein Data Bank. Protein structures were prepared for docking using AutoDock Tools, which included removing water molecules, adding hydrogen atoms, and assigning appropriate charges.

5.3 Docking Simulation:

Molecular docking was performed using AutoDock Vina software through the PyRx interface. The grid box was configured to encompass the entire protein surface to allow unbiased binding site prediction. The docking parameters were optimized to ensure comprehensive sampling of possible binding conformations. The binding affinities and molecular interactions between Spirulina-derived compounds and target proteins were analyzed and visualized using Discovery Studio software. The top-scoring poses were selected based on binding energy calculations and interaction patterns (Kondapuram *et al.*, 2021). The ligand Heme_Bc, 3-[(4-pyridin-2-yl-13-thiazol-2-yl) amino] benzoic_acid, Protoporphyrin-IX-Containing-FE, and Uridine-5'-Diphosphate were control (standard) for Peroxidase, Protein Kinase, Cytochrome P450, UDGPT protein, respectively.

5.4. Grid Dimensions:

The grid box dimension values in angstrom for 7ES0, 6TE2, 8OGI, 2IJ5, 7Z5X, and 4HWH receptors were kept at (X: 1.1595, Y:1.7280, Z: 0.7739), (X: 30.068, Y: 31.7555, Z:16.9970), (X:8.8510, Y:0.8220, Z:18.2687), (X:55.4007, Y: 87.7085, Z:187.5229), (X: 52.4281, Y: 63.0035, and Z: 58.3149). and (X: 41.2205, Y: 56.0790, and Z: 58.7571), respectively.

By setting the exhaustiveness value to eight, a series of dockings were conducted to obtain nine unique poses of the ligand molecule. Upon concluding the docking procedure, the pose that exhibited the lowest binding energy was chosen for the purpose of visualizing the interaction between the ligand and the protein through Discovery Studio 2022 [<https://10.0.142.116/pharmaceutical-sciences.543>].

5.5. ADMET Analysis:

In order to evaluate the drug-like characteristics of the natural compounds that exhibited the greatest binding affinity scores by molecular docking with ROS, and PCD proteins, ADMET STAR (<https://Immd.ecust.edu.cn/admetstar2/>) was utilized.

ADMET analysis is crucial in developing drugs and environmental risk assessment as it accurately characterizes the features of medicinal compounds and environmental contaminants. The most important pharmacokinetic parameters assessed by ADMET evaluation consist of the blood-brain barrier, CYP4502C9 activity, intestinal absorption in human, and Caco-2 permeability (Cheng *et al.*, 2012). Lipinski's rule of five parameters (RO5) states that particles must have a molecular weight below 500 g/mol, a topological polar surface area (TPSA) below 140×2 , an AlogP value below 5, number of hydrogen bond donors and acceptors below 5 and 10, respectively (Yang *et al.*, 2019, Daina *et al.*, 2017). Furthermore, the hepatotoxicity of the substances was assessed using this approach.

6- Statistical Analysis:

All experiments will be conducted in a completely randomized design with at least 10 replicates in the morphological characters and three biological replicates in the other experiment. Data will be analyzed using analysis of variance (ANOVA) followed by Tukey's HSD test was performed to determine significant differences between treatments ($p < 0.05$). Hierarchical cluster analysis was conducted using Ward's method and Euclidean distance as the similarity measure to examine clustering patterns among treatment groups. The resulting

dendrogram displayed the relationships between treatments based on both morphological and gene expression data. All the statistical analyses will be performed using SPSS software (version 18).

RESULTS AND DISCUSSION

This study investigated the potential of Spirulina extract (SpEx) to mitigate drought stress effects in wheat, integrating *in silico* analysis, morphological measurements, and gene expression data. The experimental design involved seed soaking in water (Untreated Control and drought stress groups) or SpEx (SpEx control and treatment groups) before planting, with additional foliar SpEx application to treatment groups at two weeks and 45 days after planting.

1-Morphological Characteristics:

The present study investigated the effects of drought stress (DS) and Spirulina extract (SpEx) application on the morphological characteristics of wheat plants. The results demonstrate the detrimental impact of drought stress on plant growth and the potential mitigating effects of SpEx treatment.

1.1.Plant Height:

Drought stress (DS) significantly reduced plant height by 18.2% compared to the Untreated Control group (Table 1 and Fig. 1). This finding aligns with previous studies that have reported stunted growth in wheat plants under water deficit conditions (Tardieu *et al.*, 2018). The reduction in plant height can be attributed to decreased cell elongation and division, which are common plant responses to conserve water and energy under drought stress (Farooq *et al.*, 2014).

However, application of Spirulina extract (SpEx) mitigated this effect. Plants treated with 10 mg/ml and 20 mg/ml SpEx under drought conditions reached heights of 77.5 cm and 78.2 cm, respectively, showing partial recovery towards Untreated Control levels. This protective effect of SpEx could be due to its rich composition of growth-promoting substances, including phytohormones, amino acids, and vitamins (Khan *et al.*, 2005). These bioactive compounds may help maintain cellular turgor and promote cell division and elongation, even under water-limited conditions.

Table 1: Morphological characteristics of wheat plants under different treatments, showing plant height (cm), fresh weight (g), and dry weight (g) measurements across control and experimental groups.

	Untreated Control	DS	SpEx Cont	SpEx 10 mg/ml	SpEx 20 mg/ml
Plant height	84	71.4	87.3	77.5	78.2
Fresh weight	0.64	0.45	0.75	0.69	0.71
Dry weight	0.178	0.15	0.192	0.2	0.22

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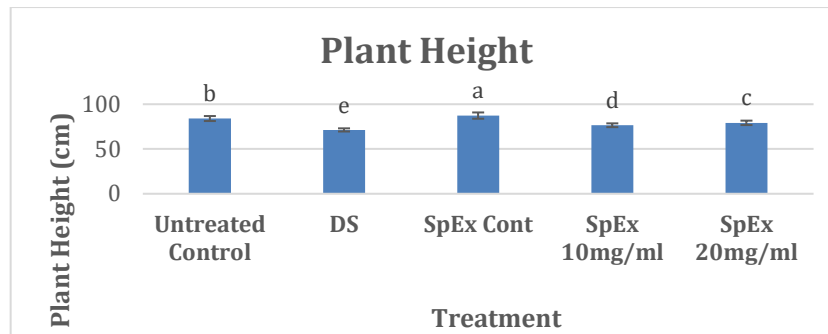


Fig. 1: Effect of drought stress and Spirulina extract treatments on wheat plant height (cm) under different experimental conditions.

Different superscript letters showed significant differences at $P < 0.05$.

1.2. Leaf Fresh Weight:

Fresh weight measurements revealed a similar pattern. DS reduced fresh weight 29.7% relative to the Untreated Control. This substantial decrease in biomass is likely due to reduced photosynthetic capacity and increased leaf senescence under drought conditions (Anjum *et al.*, 2011).

While SpEx treatments under drought conditions resulted in fresh weights of 0.69 g (10 mg/ml SpEx) and 0.71 g (20 mg/ml SpEx), indicating a protective effect. Interestingly, the SpEx control group exhibited the highest fresh weight at 0.75 g, suggesting potential growth-promoting effects of SpEx even under non-stress conditions (Table 1 and Fig. 2).

The application of SpEx showed promising results in mitigating drought-induced biomass loss. Plants treated with 10 mg/ml and 20 mg/ml SpEx under drought conditions exhibited fresh weights closer to the Untreated Control group. This protective effect may be attributed to the antioxidant properties of Spirulina, which can help plants cope with oxidative stress induced by drought (Abd El-Baky *et al.*, 2009, Abd El-Baky *et al.*, 2014).

Notably, the SpEx control group displayed the highest fresh weight, suggesting that Spirulina extract may have growth-promoting effects even under non-stress conditions. This observation is consistent with studies reporting the biostimulant properties of microalgae extracts in various crop species (Chiaiese *et al.*, 2018).

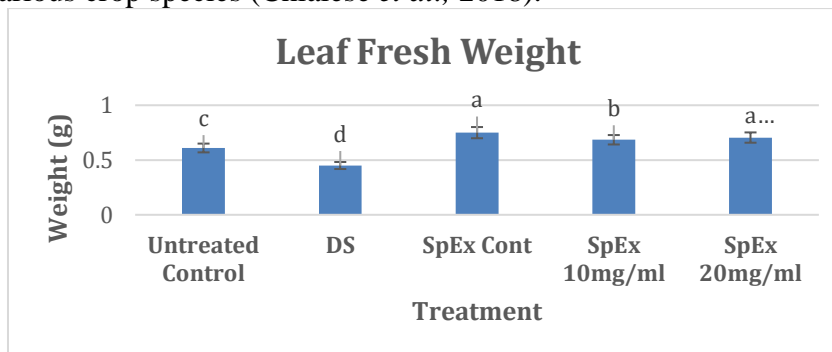


Fig. 2: Impact of drought stress and Spirulina extract applications on wheat leaf fresh weight (g). Different superscript letters showed significant differences at $P < 0.05$.

1.3. Dry Weight:

The dry weight measurements provided further evidence of SpEx's beneficial effects on wheat plants under drought stress. Dry weight data further supported the beneficial effects of SpEx. While DS reduced dry weight by 15.7%, compared to the Untreated Control, both 10 mg/ml and 20 mg/ml SpEx treatments under drought resulted in increased dry weights of 0.2 g and 0.22 g, respectively. This suggests that SpEx not only mitigates the negative effects of drought but may also enhance biomass accumulation (Table 1 and Fig. 3).

The observed increase in dry weight could be attributed to improved photosynthetic efficiency and enhanced nutrient uptake facilitated by SpEx application. Spirulina extract has been shown to contain various minerals, including potassium, calcium, and magnesium, which play crucial roles in plant metabolism and stress tolerance (Godlewska *et al.*, 2019).

In conclusion, this study demonstrates the potential of Spirulina extract as a biostimulant to alleviate drought stress effects on wheat plants. The application of SpEx improved plant height, fresh weight, and dry weight under water-limited conditions, indicating its ability to enhance drought tolerance in wheat.

Future research should focus on elucidating the specific mechanisms by which SpEx confers drought tolerance, such as its effects on antioxidant enzyme activities, osmolyte accumulation, and gene expression patterns related to stress response. Additionally, field trials are necessary to validate the efficacy of SpEx application under varying environmental conditions and to optimize application rates and timing for maximum benefit.

These findings contribute to the growing body of research on sustainable agricultural practices and may have important implications for improving crop resilience in the face of climate change-induced drought events.

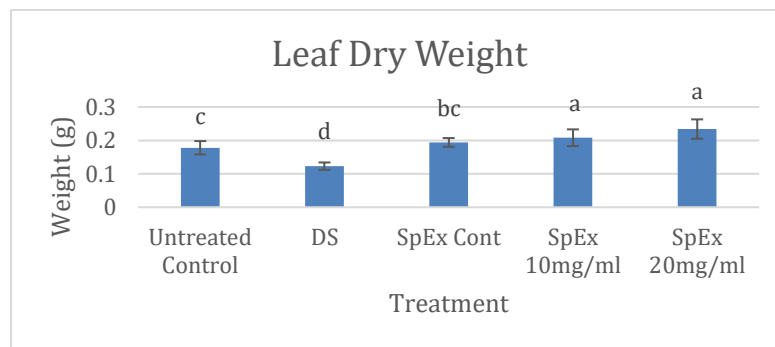


Fig. 3: Changes in wheat leaf dry weight (g) in response to drought stress and varying concentrations of Spirulina extract.

Different superscript letters showed significant differences at $P < 0.05$.

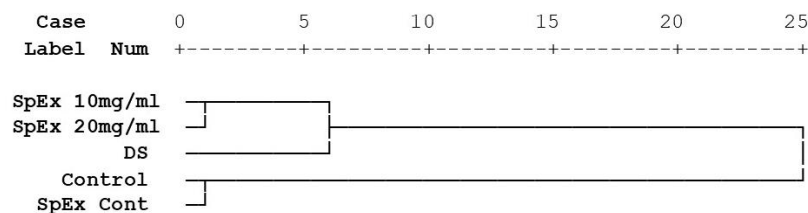


Fig. 4: Hierarchical cluster analysis dendrogram based on morphological characteristics of wheat plants under different treatments.

1.4. Morphological Characters Dendrogram:

The dendrogram of wheat plant morphological characteristics (Fig. 4) reveals clear clustering patterns across control and treated groups, showing how drought stress (DS) and Spirulina treatments affect growth. Drought stress caused notable reductions in plant height, fresh weight, and dry weight, aligning with studies indicating that water deficit can limit cell elongation and division, thereby reducing plant biomass and height (Tardieu *et al.*, 2018; Farooq *et al.*, 2014). In contrast, Spirulina extract (SpEx) treatments at 10 mg/ml (SpEx 10) and 20 mg/ml (SpEx 20) improved these growth parameters under drought, clustering closer to the untreated control group. This suggests that Spirulina extract can help plants resist drought stress by enhancing cellular turgor and mitigating oxidative damage, likely due to the bioactive components in Spirulina, such as phytohormones, amino acids, and antioxidants (Khan *et al.*, 2005, Chiaiese *et al.*, 2018).

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In non-stressed conditions, the SPEx control group (seeds pre-treated with Spirulina without subsequent stress) forms a distinct cluster, indicating that Spirulina treatment promotes growth even in favorable environments. This growth-promoting effect is consistent with studies showing the biostimulant properties of Spirulina and other algae-derived extracts in enhancing plant performance through mechanisms such as improved nutrient uptake and modulation of growth-related pathways (Abd El-Baky *et al.*, 2009, Chiaiese *et al.*, 2018).

2-Gene Expression Analysis Under Drought Stress and Spirulina Extract Treatment:

The present study investigated the effects of drought stress (DS) and Spirulina extract (SpEx) treatment on the expression of four stress-responsive genes (UDPGT, Protein kinase, Cytochrome P450, and Peroxidase gene) in wheat plants.

2.1.UDPGT Gene Expression:

Our results show a 2.23-fold upregulation of UDPGT under drought stress. This expression was further enhanced by SpEx treatments, with 10 mg/ml and 20 mg/ml SpEx resulting in 2.93-fold and 3.67-fold increases, respectively (Table 2 and Fig. 5).

Previous research has demonstrated that transgenic tobacco plants expressing the human *UDPGT* gene exhibited increased cell wall thickness and chlorophyll content, contributing to enhanced drought tolerance and improved dry weight accumulation (Khalil *et al.*, 2010). Furthermore, studies in *Arabidopsis* showed that *HvUGT1* overexpression conferred superior drought tolerance, enhanced water retention, reduced cellular damage, and elevated flavonoid accumulation (Feng *et al.*, 2024). Similarly, the overexpression of *UGT85E1* in transgenic rice enhanced drought tolerance by promoting stomatal closure and *ABA* accumulation (Liu *et al.*, 2021). The observed *UDPGT* upregulation in our study aligns with previous findings that reported elevated UDPGT expression under abiotic stress conditions (Li *et al.*, 2017). The additional enhancement in expression following SpEx treatment suggests that Spirulina extract contains compounds that may function as elicitors, further activating the plant's stress response mechanisms."

Table 2: Relative gene expression analysis of stress-response genes (*UDPGT*, Protein Kinase, Cytochrome P450, and Peroxidase) under various treatment conditions (drought stress, and Spirulina treatments).

	Untreated Control	DS	SpEx Cont	SpEx 10 mg/ml	SpEx 20 mg/ml
<i>UDPGT</i>	1	2.23	1.27	2.93	3.67
Protein Kinase	1	2.7	1.36	3.57	4.56
Cytochrome P450	1	2.83	1.32	4.03	5.23
Peroxidase	1	3.4	1.41	4.67	6.13

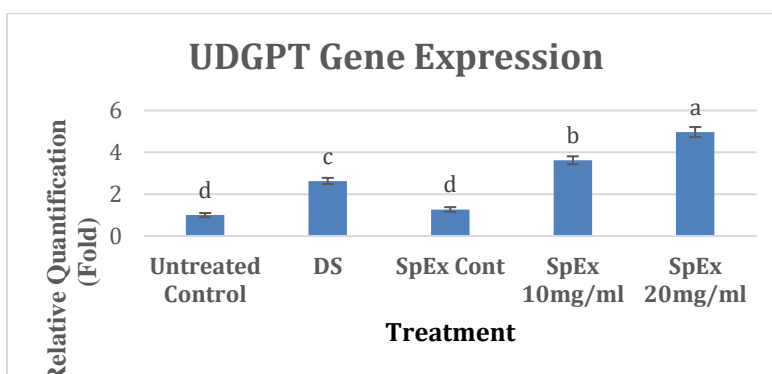


Fig. 5: Expression levels of UDPGT gene in wheat plants under various treatment conditions. Different superscript letters showed significant differences at $P < 0.05$.

2.2. Protein Kinase Gene Expression:

Protein kinases are key regulators of cellular processes and stress signaling in plants (Ye *et al.*, 2017). Protein Kinase gene expression increased 2.7-fold under DS (Table 2 and Fig. 6). SpEx treatments amplified this response, with 3.57-fold and 4.56-fold increases observed for 10 mg/ml and 20 mg/ml concentrations, respectively, indicates a robust activation of stress signaling cascades. This upregulation may contribute to improved drought tolerance by modulating various downstream stress response mechanisms, including osmolyte accumulation and antioxidant defense systems (Zhu, 2016).

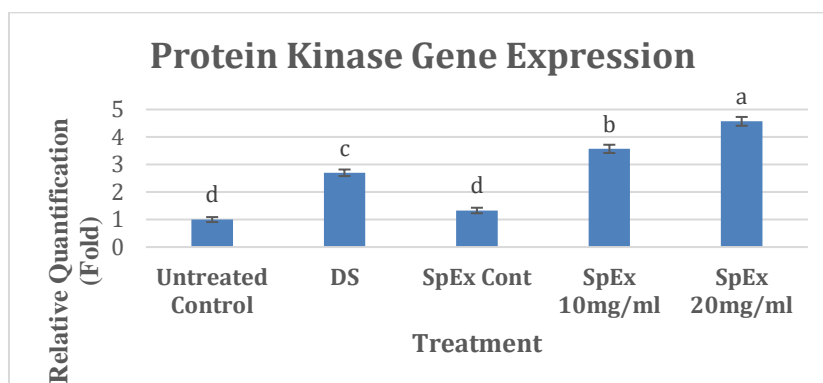


Fig. 6: Analysis of Protein Kinase gene expression under drought stress and Spirulina extract treatments in wheat plant. Different superscript letters showed significant differences at $P < 0.05$.

2.3. Cytochrome P450 Gene Expression

Cytochrome P450 exhibited a 2.83-fold upregulation under DS. SpEx treatments led to more pronounced increases, with 4.03-fold and 5.23-fold changes for 10 mg/ml and 20 mg/ml applications, respectively (Table 2 and Fig. 7).

Cytochrome P450 enzymes are involved in the biosynthesis of various secondary metabolites and plant hormones, playing important roles in stress adaptation (Pandian *et al.*, 2020). The upregulation of this gene under drought stress and the further increases with SpEx treatments suggest enhanced secondary metabolism and hormone biosynthesis. This may contribute to improved stress tolerance through the production of protective compounds and fine-tuning of hormone-mediated stress responses (Xu *et al.*, 2015).

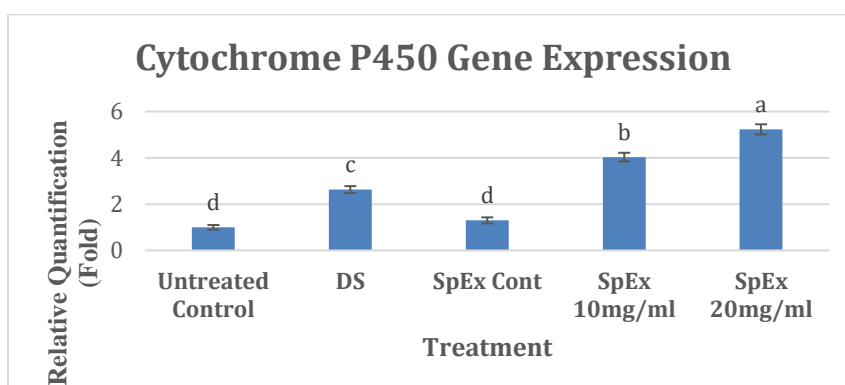


Fig. 7: Cytochrome P450 gene expression levels in wheat under different conditions. Different superscript letters showed significant differences at $P < 0.05$.

2.4. Peroxidase Gene Expression:

Peroxidase showed the most dramatic changes among the genes studied. DS induced a 3.4-fold increase in expression, while SpEx treatments further boosted expression to 4.67-

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fold and 6.13-fold for 10 mg/ml and 20 mg/ml concentrations, respectively (Table 2 and Fig. 8).

Peroxidases are key enzymes in the plant's antioxidant defense system, crucial for mitigating oxidative stress induced by drought (Sharma *et al.*, 2012). The dramatic upregulation of peroxidase gene expression under drought stress and its further enhancement with SpEx treatments indicate a potent activation of antioxidant mechanisms. This increased peroxidase expression may lead to more efficient scavenging of reactive oxygen species (ROS), thereby reducing oxidative damage and improving drought tolerance (Sofo *et al.*, 2015).

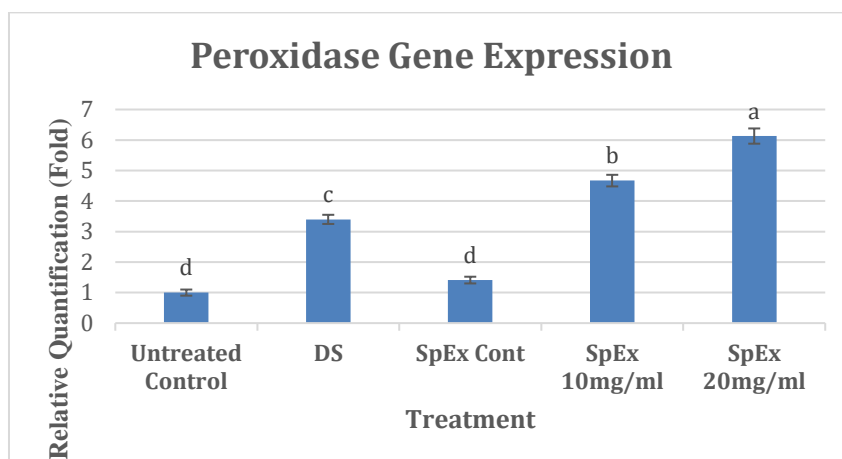


Fig. 8: Peroxidase gene expression profiles in response to drought stress and Spirulina extract applications. Different superscript letters showed significant differences at $P < 0.05$.

2.5. Gene Expression Profile Dendrogram:

In the gene expression-based dendrogram (Fig. 9) levels of UDPGT, protein kinase, cytochrome P450, and peroxidase genes, reveal distinct clustering of DS and Spirulina-treated groups. In DS plants, these genes were significantly upregulated, forming a unique cluster that differs from the untreated control, which is consistent with previous findings that drought stress activates genes involved in signaling, detoxification, and oxidative stress response (Li *et al.*, 2017, Zhu, 2016). Spirulina-treated groups (SPEX 10 and SPEX 20) clustered between the DS and control groups, showing even higher upregulation of these genes than DS alone, suggesting that Spirulina extract may act as an elicitor of stress tolerance pathways.

The enhanced gene expression in Spirulina-treated plants could indicate that Spirulina compounds play a role in priming stress-response pathways, improving the plants' antioxidant defense mechanisms and metabolic stability under drought (Godlewska *et al.*, 2019; Zhang *et al.*, 2021). Moreover, the SPEX control group under non-stress conditions displayed slight upregulation of these genes, which places it between untreated control and DS groups in the clustering analysis. This "priming" effect, where mild exposure to certain biostimulants prepares the plant to handle future stress better, has been widely documented in biostimulant studies (Savvides *et al.*, 2016). Spirulina's priming ability may be linked to its polysaccharides, peptides, and phytohormones, which are known to activate stress response pathways even under non-stressed conditions (Godlewska *et al.*, 2019).

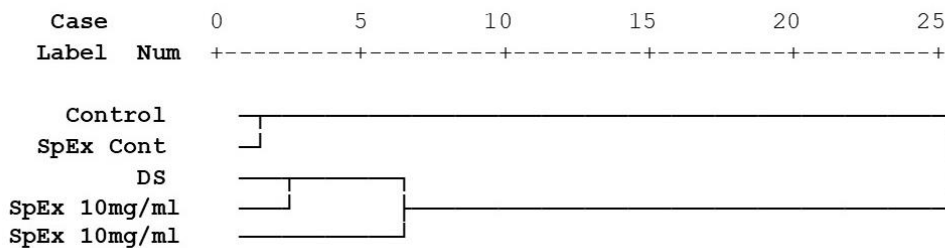


Fig. 9: Hierarchical cluster analysis dendrogram based on gene expression profile data of all studied genes (UDPGT, Cytochrome P450, Protein Kinase, and Peroxidase genes).

2.6. SpEx Priming Effect:

Interestingly, the SpEx control group exhibited slight upregulation of all four genes (1.27 to 1.41-fold) compared to the untreated control. This observation suggests that SpEx may have a priming effect on wheat plants, activating stress response pathways even under non-stress conditions. Priming is a phenomenon where plants exposed to mild stress or certain compounds become more resistant to future stress events (Savvides *et al.*, 2016). The priming effect of SpEx could be attributed to various bioactive compounds present in *Spirulina*, such as polysaccharides, peptides, and phytohormones, which have been reported to elicit plant defense responses (Godlewska *et al.*, 2019).

In conclusion, this study demonstrates that *Spirulina* extract treatment enhances the expression of key stress-responsive genes in wheat under drought conditions. UDPGT, protein kinase, cytochrome P450, and peroxidase gene upregulation suggest a multi-faceted molecular response, potentially contributing to improved drought tolerance. Moreover, the observed priming effect of SpEx under non-stress conditions indicates its potential as a preventive treatment to enhance stress resilience in wheat.

Future research should focus on:

1. Investigating the specific components of SpEx responsible for gene expression modulation.
2. Exploring the downstream effects of enhanced gene expression on metabolite profiles and physiological parameters.
3. Conducting field trials to validate the efficacy of SpEx in improving drought tolerance under varying environmental conditions.
4. Examining the long-term effects of SpEx priming on plant performance and stress tolerance.

These findings contribute to our understanding of plant-biostimulant interactions and open new avenues for developing sustainable strategies to enhance crop resilience in the face of climate change-induced drought events.

3-Gas Chromatography (GC) Mass Analysis:

Analysis of the *Spirulina* extract (SpEx) using gas chromatography-mass spectrometry (GC-MS) revealed the presence of 27 distinct bioactive compounds (Table 3).

4-*In silico* Study of the UDPGT, Cytochrome P450, Protein Kinase, and Peroxidase Proteins:

The UDPGT, Protein Kinase, Peroxidase, and Cytochrome P450 gene products play crucial roles in various cellular processes including stress response and xenobiotic metabolism. The molecular interaction analysis between *Spirulina*-derived compounds and these proteins revealed that these compounds displayed lower binding affinities compared to the control ligand (Table 3). These reduced affinities suggested that the interactions between these *Spirulina*-derived ligands and the receptors are relatively weak, potentially impacting their effectiveness in inhibiting or activating these proteins within relevant

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biological pathways. However, while the binding strength is lower, these compounds might still serve as viable options due to their potential benefits, such as reduced toxicity or minimized side effects, which can be advantageous in biological applications and therapeutic contexts (Wang *et al.*, 2017 (a)).

Table 3: Molecular Docking Results of Peroxidase, Protein Kinase, Cytochrome P450, and UDPGT proteins Interactions With Spirulina-Derived Compounds, Displaying Binding Affinity Scores (RMSD/UB and RMSD/LB = 0). Control was highlighted.

Ligand	Binding Affinity			
	Peroxidase	KINASE	CYP450	UDPGT
Control				
HEME_BC	-8.4	-	-	-
3-[(4-pyridin-2-yl-13-thiazol-2-yl)amino]benzoic_acid	-	-9.4	-	-
PROTOPORPHYRIN-IX-CONTAINING-FE	-	-	-10.8	-
URIDINE-5'-DIPHOSPHATE	-	-	-	-9.7
Spirulina Extract (SpEx)				
1,13-tetradecadien-3-one	-5.3	-6.6	-5.7	-5.4
11-Octadecenoic-acid methyl-ester	-5.5	-6.3	-6.1	-5.4
17-pentatriacontene	-1.5	-1.1	-1.1	-1
2-Hexadecanol	-5.6	-5.7	-5.7	-5.3
2-hydroxy-246-trimethoxychalcone	-7.8	-6.3	-7.6	-7.6
2-pentadecanone61014-trimethyl-	-6	-8.7	-5.9	-6
9,12,15-Octadecatrienoic-acid (ZZZ)-	-6.1	-7.1	-6.7	-6.3
9,12-Octadecadienoic-acid (ZZ)-methyl-ester	-5.8	-7.3	-6.4	-5.4
9-Hexadecanoic-acid methyl-ester(z)-	-5.5	-6.9	-5.7	-5.7
9-octadecen-1-ol(z)-	-5.2	-6.7	-5.3	-5.2
Araguspongins	-8.9	-6.6	-10.8	-9.3
Batilol	-5.3	-9.1	-6.1	-5.6
Casticin	-7.1	-6.3	-7.5	-8.1
Gamolonic -acid	-6.6	-8.4	-6.1	-6
Heptadecane	-5.1	-6.6	-6.9	-5.8
Hexadecanoic_acid_ethyl_ester	-5.2	-7.1	-5.4	-5.3
Hexadecenoic-acid methyl-ester	-5.3	-6	-6	-5
Linoleic_acid_ethyl_ester	-5.8	-6.2	-5.8	-5.3
Lupeol	-8.4	-6.3	-6	-5.9
methyl-13-methyltetradecanoate	-5.1	-6.8	-9.6	-9.3
Neophytadiene	-5.9	-8.7	-5.8	-5.5
Octadecanoic-acid	-5.4	-6.2	-6.2	-6.2
Phytol	-6.3	-7.3	-5.3	-5.2
Silybin	-8.3	-6.6	-6.8	-6
Strophanthidine	-7.3	-7.5	-10.2	-9.5
Tetradecanal	-5.4	-9.3	-9.3	-8.9
Tetradecanoic-acidethyl-ester	-5.5	-7.8	-5.3	-5.1

4.1. *In silico* Study of The ROS and PCD Proteins:

To evaluate the potential interactions between the active SpEx compounds and stress-related proteins, we conducted an *In Silico* study focusing on Reactive Oxygen Species (ROS) and Programmed Cell Death (PCD) associated proteins. This aligns with previous studies that have reported a diverse array of bioactive compounds in *Spirulina*, including phenolics, flavonoids, and other antioxidants (Soni *et al.*, 2017). The presence of these compounds contributes to the multifaceted effects of *Spirulina* on plant stress responses.

For this analysis, we utilized a control compound that was previously established to bind to the ROS and PCD proteins in the molecular docking database. This control served as a benchmark for comparing the binding affinities of the SpEx compounds.

Our computational analysis identified four compounds from the SpEx that exhibited notable binding affinities to the receptor sites of both ROS and PCD-related proteins (Table 4). These compounds were Araguspongin, Lupeol, Silybin, and Strophanthidine. Each compound demonstrated varying degrees of interaction with both protein types.

For the ROS-associated protein, the binding affinity scores were as follows: Araguspongin (8.3), Lupeol (7.7), Silybin (8.1), and Strophanthidine (7.6). Similarly, for the PCD-related protein, the scores were: Araguspongin (8.1), Lupeol (7.6), Silybin (7.9), and Strophanthidine (7.5). These binding affinities exceeded those of the control compound, suggesting potential effectiveness in modulating stress-related processes. This aligns with previous studies that had identified bioactive compounds in algal extracts with stress-mitigating properties (Kumar *et al.*, 2024).

Araguspongin consistently displayed the highest binding affinity for both protein types, closely followed by Silybin then Lupeol, and the last one was Strophanthidine. This suggests these compounds may have significant potential for modulating ROS and PCD-related processes in plants under stress conditions.

Araguspongin exhibited the highest binding affinity for both ROS (8.3) and PCD (8.1) related proteins. While Araguspongin is less commonly discussed in plant stress literature, its strong binding affinity suggests it may be a potent modulator of stress response pathways. Future studies should investigate its specific effects on plant stress tolerance.

Lupeol, with binding scores of 7.7 (ROS) and 7.6 (PCD), has been previously reported to possess antioxidant properties in plants. For instance, Zia *et al.* (2023) found that Lupeol treatment enhanced antioxidant enzyme activities in *Brassica nigra* under salt stress. Our *in silico* results support these findings and suggest that Lupeol may directly interact with ROS and PCD-related proteins to mediate its protective effects.

Silybin, showing binding scores of 8.1 (ROS) and 7.9 (PCD), is known for its strong antioxidant properties. While more commonly studied in mammalian systems, recent research has begun to explore its effects in plants. For example, Guo *et al.* (2018) reported that Silybin application mitigated oxidative stress in wheat under drought conditions. Our results provide a potential molecular basis for these observations, suggesting direct interactions between Silybin and stress-related proteins.

Strophanthidine, with binding scores of 7.6 (ROS) and 7.5 (PCD), has been less studied in the context of plant stress responses. However, its structural similarity to other cardiac glycosides suggests potential roles in ion homeostasis and membrane stability (Waadt *et al.*, 2022), which could indirectly influence ROS and PCD pathways.

To further elucidate the nature of these interactions, we generated 2D molecular interaction diagrams. These visual representations, depicted in Figures 10 and 11, illustrate the specific binding modes between the identified compounds and the receptor sites of the ROS and PCD proteins, respectively. The diagrams elucidate the specific amino acid residues involved in the interactions, the types of bonds formed, and the spatial arrangement of the ligands within the protein binding pockets. Furthermore, Table 5 provides a systematic breakdown of the various interaction types observed in this study, including hydrogen

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bonding, hydrophobic interactions, and other non-covalent forces, which are crucial for the specificity and strength of protein-ligand interactions (Du *et al.*, 2016). This comprehensive analysis not only visualizes the binding modes, but also quantifies the molecular forces driving the affinity between the SpEx compounds and the stress-related proteins, offering valuable insights into the potential mechanisms of action of these bioactive components.

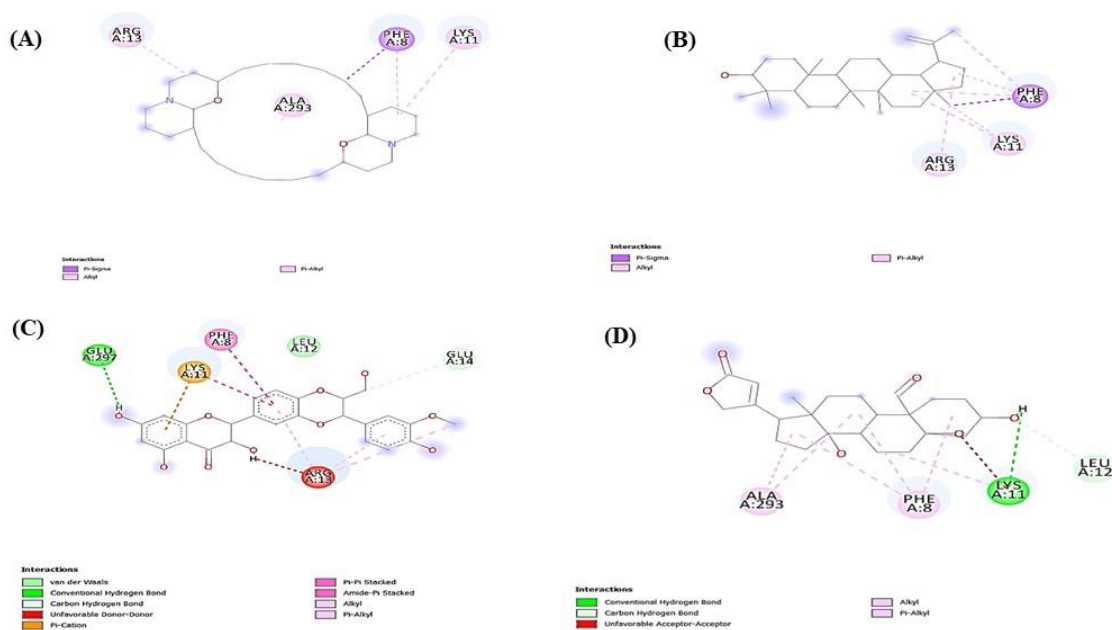


Fig. 10: 2D molecular interaction of ROS with (A) Araguspongin, (B) Lupeol, (C) Silybin, and (D) Strophanthidine

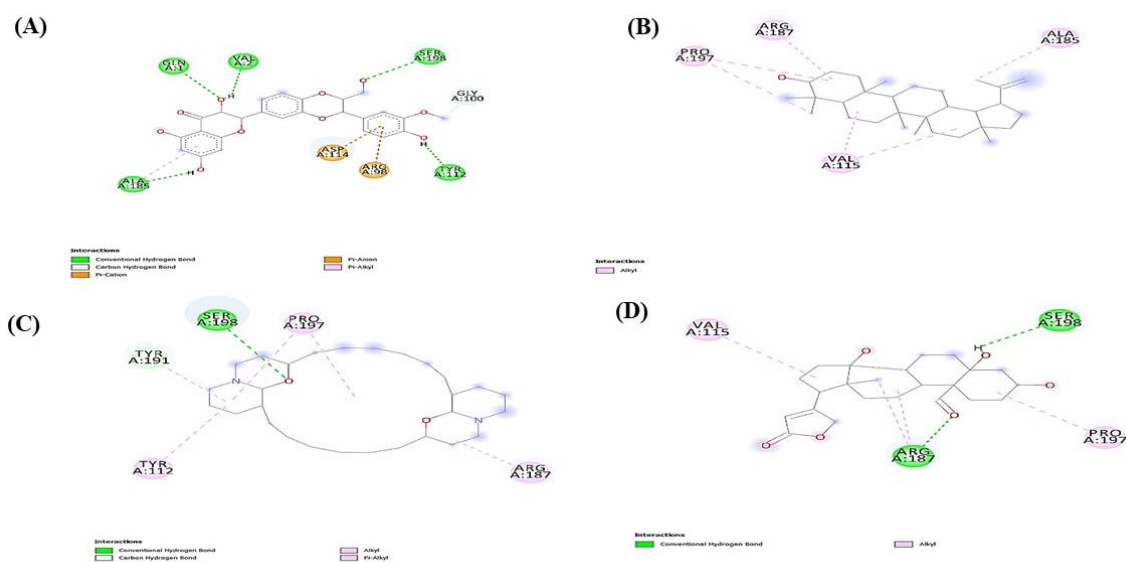


Fig. 11: 2D molecular interaction of PCD with (A) Araguspongin, (B) Lupeol, (C) Silybin, and (D) Strophanthidine

While these *in silico* results are promising, it's important to note that computational predictions require experimental validation. Therefore *in vivo* experiment to confirm the predicted interactions and their physiological relevance in plant stress responses was carried out.

In conclusion, this *in silico* study provides valuable insights into the potential molecular mechanisms by which Spirulina extract may modulate plant stress responses. The identification of specific compounds with high binding affinities to ROS and PCD-related proteins offers new avenues for targeted investigations into the stress-mitigating properties of Spirulina and its constituents in plants.

Table 4: Analysis of binding affinities between ROS and PCD proteins with selected high-scoring compounds, including control values.

Ligand	Binding affinity ROS	Binding affinity PCD	RMSD/UB	RMSD/LB
Control	-7.3	-4.1	0	0
Araguspongin	-8.3	-8.1	0	0
Lupeol	-7.7	-7.6	0	0
Silybin	-8.1	-7.9	0	0
Strophanthidine	-7.6	-7.5	0	0

Table 5: Type of interaction and residue position between docked ROS and PDC proteins and compounds.

Protein	Ligand	Interaction	Residue and Position	
ROS	Araguspongin	PI-Sigma	PHE A:8	
		Alkyl/Pi-Alkyl	ALA A:293, LYS A:11, and ARG A:13	
	Lupeol	PI-Sigma	PHE A:8	
		Alkyl/Pi-Alkyl	LYS A:11, and ARG A:13	
	Silybin	Conventional	GLU A:297	
		PI-Cation	LYS A:11	
		Pi-Pi Stacked/Amide- Pi Stacked	PHE A:8	
		Van der waals	LEU A:12	
	Strophanthidine	Carbon	GLU A:14	
		Carbon	LEU A:12	
Conventional		LYS A:11		
PCD	Araguspongin	Alkyl/Pi-Alkyl	ARG A:187, TYR A:112, and PRO A:197	
		Conventional	SER A:198	
		Carbon	TYR A:191	
	Lupeol	Alkyl	ALA A:185, VAL A:115, ARG A:187, and PRO A:197	
		Silybin	Conventional	TYR A:112, SER A: 198, VAL A:2, GLN A:1, and ALA A:185
			Carbon	GLU A:100
	Strophanthidine	PI-Cation/PI-Anion	ARG A:98, and ASP A:114	
		Conventional	ARG A:187, and SER A:198	
		Alkyl	PRO A:197, and VAL A:115	

4.2.ADMET Properties:

The ADMET (Absorption, Distribution, Metabolism, Excretion, and Toxicity) properties analysis revealed distinct characteristics among the four compounds: Lupeol, Araguspongin, Silybin, and Strophanthidine. These properties provide crucial insights into their potential therapeutic applications and limitations (Table 6).

The ADMET properties of the Spirulina-derived compounds further validated their suitability for bioactivity, with all tested compounds adhering to Lipinski's Rule of Five. For instance, Araguspongin and Lupeol displayed favorable GI absorption and low toxicity, along with adequate bioavailability scores, indicating potential utility as safe bioenhancers in plant stress tolerance applications. The molecular weight, hydrogen bond profiles, and TPSA values across compounds supported high cellular permeability without disrupting

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membrane integrity (6). Notably, Araguspongin's high blood-brain barrier permeability, as seen in ADMET, suggests its effectiveness in crossing cellular barriers. These characteristics align with previous studies on bioactive compound distribution (Daina *et al.*, 2017)

Table 6: Comprehensive ADMET properties analysis of selected bioactive compounds (Lupeol, Araguspongin, Silybin, and Strophanthidine).

ADMET	Lupeol	Araguspongin	Silybin	Strophanthidine
Classification	Anti-inflammatory, Antioxidant, Anticancer, Antimicrobial, Hepatoprotective, and Cardioprotective	Anti-HIV, Antiviral, Anticancer, Cytotoxic, Anti-inflammatory, Antifungal and Antibacterial	Flavonolignan, antioxidant, antineoplastic, Hepatoprotective Agent, Antioxidant, Anti-inflammatory, Anticancer, Antifibrotic, and Antidiabetic	Cardiotonic Agent, Toxic Compound, and Na ⁺ /K ⁺ -ATPase Inhibitor
Molecular weight (g/mol)	426.72	446.71	-4.14	404.50
Number of H-bond acceptors	1	4	10	6
Number of H-bond donors	1	0	5	3
Water solubility (logS)	-6.74	-4.31	-4.14	-2.62
TPSA (Å²)	20.23	24.94	155.14	104.06
Molar refractivity (MR)	135.14	141.93	120.55	106.16
Log Kp (skin permeation) cm/s	-1.90	-3.79	-7.89	-8.31
Bioavailability score	0.55	0.55	0.55	0.55
GI absorption	Low	High	Low	High
Lipinski rule	Yes	Yes	Yes	Yes
Blood-Brain Barrier (BBB)	No	Yes	No	No
CYP1A2 inhibitor	No	No	No	No
CYP2C19 inhibitor	No	No	No	No
CYP2C9 inhibitor	No	No	No	No
CYP2D6 inhibitor	No	No	No	No
CYP3A4 inhibitor	No	No	No	No

Conclusion

This comprehensive investigation provides compelling evidence for Spirulina extract's effectiveness in enhancing wheat drought tolerance through multiple mechanisms. Following the experimental progression, our findings reveal several key insights:

Morphologically, SpEx treatment significantly improved plant responses to drought stress, with notable increases in plant height, fresh weight, and dry weight. The hierarchical cluster analysis of morphological traits demonstrated clear differentiation between SpEx-treated and untreated plants, suggesting distinct physiological adaptations under stress conditions.

Gene expression analyses revealed significant upregulation of all four studied stress-responsive genes, with Peroxidase showing the most dramatic increase (6.13-fold with 2% SpEx). This enhanced expression profile indicates robust activation of stress defense mechanisms, particularly antioxidant systems and secondary metabolism pathways. The observed priming effect in SpEx-treated plants under non-stress conditions suggests potential preventive applications.

The molecular docking studies, conducted to understand the mechanistic basis of these observed effects, revealed significant interactions between SpEx compounds and six key proteins: UDPGT, Cytochrome P450, Peroxidase, Protein kinase, and notably, ROS and PCD-related proteins. These computational analyses provided structural insights into how SpEx compounds might modulate stress response pathways at the molecular level. The timing of these docking studies after experimental observations allowed for targeted investigation of the molecular mechanisms underlying the observed biological effects.

Further analysis of ROS and PCD protein interactions through ADMET studies identified four promising compounds (Araguspongin, Lupeol, Silybin, and Strophanthidine) with favorable bioavailability and safety profiles. This additional layer of analysis strengthens the potential for practical applications of these compounds in agricultural settings.

These findings contribute significantly to our understanding of plant-biostimulant interactions and offer practical implications for sustainable agriculture. Future research should focus on:

1. Field-scale validation of SpEx applications under varying environmental conditions.
2. Investigation of potential synergistic effects between identified bioactive compounds.
3. Development of optimized formulations based on the most effective SpEx components.
4. Long-term studies on the stability and persistence of SpEx-induced stress tolerance.

The integration of experimental and computational approaches in this study provides a robust foundation for developing effective, sustainable solutions to enhance crop resilience against drought stress.

Declarations:

Ethical Approval: No plant, animal model(s) or human subjects were recruited directly for the current study. Consequently, no ethical considerations are necessary.

Conflict of interest: The authors declare no conflict of interest.

Authors Contributions: I hereby verify that all authors mentioned on the title page have made substantial contributions to the conception and design of the study, have thoroughly reviewed the manuscript, confirm the accuracy and authenticity of the data and its interpretation, and consent to its submission.

Funding: The author(s) received no specific funding for this work.

Availability of Data and Materials: All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request.

Acknowledgments: We would like to express our appreciation to Professor Dr. Ashraf Bakry Abdelrazik for supplying the spirulina that was crucial for conducting this study.

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