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RESEARCH ARTICLE
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Optimizing Salinity and Drought Stress Elicitation to Enhance Phytochemical Accumulation and Antioxidant Activity in *Rhinacanthus nasutus* (L.) Kurz calli

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ABSTRACT

Rhinacanthus nasutus is a medicinal plant valued for its antioxidant and pharmacological properties, largely attributed to phenolic and flavonoid contents. This study enhanced bioactive metabolite production in cultures of *R. nasutus* calli through abiotic elicitation using sodium chloride (NaCl) and polyethylene glycol (PEG 6000). Calli was produced from nodal on the Murashige and Skoog (MS) medium added with 1mg L^{-1} kinetin and 1mg L^{-1} 2,4-D, and elicited with varying concentrations of NaCl (0-200mM) and PEG (0-4%) for 7 days, followed by a 2-week recovery period. Growth parameters, physiological traits, pigment content, total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity were evaluated. Results demonstrated that moderate stress conditions significantly improved biomass accumulation, with the highest fresh weight (1.82±0.21g) at 4% PEG combined with 100mM NaCl. Severe stress (4% PEG + 200mM NaCl) intensified oxidative stress, as indicated by increased malondialdehyde and electrolyte leakage. The highest TPC (26.96±0.69mg GAE g^{-1}) occurred under 0 % PEG + 150mM NaCl, while the highest TFC (17.08±0.86mg QE g^{-1}) was recorded at 0 % PEG + 100mM NaCl. The optimal condition for enhanced antioxidant activity was 1 % PEG + 100-150mM NaCl (FRAP = 33.40±0.53mg TE g^{-1}; DPPH IC_{50} = 0.41±0.00mg mL^{-1}; ABTS IC_{50} = 0.14±0.00mg mL^{-1}). These findings demonstrated that controlled abiotic elicitation optimized phenolic and flavonoid biosynthesis, enhancing the antioxidant potential of *R. nasutus* calli for pharmaceutical applications.

Keywords: Abiotic elicitation, Antioxidant activity, Callus culture, Phenolic, Flavonoid.

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INTRODUCTION

Rhinacanthus nasutus is a medicinal plant renowned for its pharmacological anti-inflammatory, antioxidant, and antimicrobial effects. These therapeutic benefits are primarily attributed to bioactive compounds such as phenolic acids, flavonoids, and alkaloids, which are utilized in traditional medicine for a variety of purposes (Saboon et al., 2019). The *R. nasutus* is a highly valued ethnobotanical species widely used in traditional medicine across Southeast Asia for treating various ailments, including skin diseases, diabetes, and inflammation (Irawan et al., 2025). Its therapeutic efficacy is strongly linked to its comprehensive array of bioactive compounds. The dominant class of compounds isolated from the roots, stems, and leaves are the naphthoquinones (e.g., rhinacanthin-C, -D, and -N), but the pharmacological profile is synergistically supported by various other constituents, including phenolic acids, lignans, and flavonoids (Senthilkumaran & Saranya, 2022). The demand for these bioactive compounds in pharmaceutical and nutraceutical applications has spurred significant interest in methods to enhance their production. One effective strategy for optimizing the synthesis of these metabolites is through *in vitro* cultivation systems. Callus cultures offer a controlled and easily manipulated environment, promoting the production of secondary metabolites, often difficult to achieve with whole plants (Fazili et al., 2022).

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Elicitation is a method of exposing plants to biotic or abiotic stimuli in order to increase the production of secondary metabolites. Various elicitors, including both biotic and abiotic stress agents, have been shown to enhance metabolite production by activating stress response pathways in plants (Humbal & Pathak, 2023). Sodium chloride (NaCl) and polyethylene glycol (PEG 6000) are commonly used as abiotic elicitors. NaCl induces osmotic stress, triggering the plant to produce compounds that help it cope with the stress. PEG 6000 simulates drought stress, activating the plant's defense mechanisms to promote the production of secondary metabolites like phenolics, flavonoids, and other antioxidants (Asaad et al., 2022). These compounds contribute to the plant's antioxidant capacity, and their production is closely linked to the pharmacological efficacy of *R. nasutus*.

Biotechnological enhancement strategies are fundamentally based on the concept of hormesis, where a mild, non-lethal stressor is applied to induce an over compensatory physiological defense response (Sperdouli et al., 2023). Within plant cell cultures, this mild stress, or elicitation, triggers a crucial internal signaling cascade. Many forms of abiotic stress, including the cellular perturbation caused by polyploidy induction, rapidly lead to the production of Reactive Oxygen Species (ROS). Among these, hydrogen peroxide H_2O_2 is not merely a toxic byproduct but functions as a critical secondary messenger that coordinates the cell's adaptive response (Iqbal et al., 2025). H_2O_2-mediated signaling is known to regulate stress-responsive gene expression, activate antioxidant defense systems (such as SOD and CAT), and promote the accumulation of protective compounds like osmolytes and phenolic antioxidants (Khan et al., 2025). This deliberate imposition of stress is therefore a powerful tool to redirect metabolic flux towards the synthesis of defense-related, high-value secondary metabolites.

The successful up-regulation of phenolic and flavonoid biosynthesis holds clear pharmacological implications. The primary naphthoquinones and the synergistically acting phenols provide the plant with its renowned protective capabilities. Studies utilizing contemporary analytical methods (UHPLC-MS/MS QTOF) confirm that the leaf extracts possess significant free radical scavenging activity and exhibit promising anti-diabetic potential via the inhibition of the dipeptidyl peptidase IV (DPP IV) enzyme, a key target in type 2 diabetes management (Irawan et al., 2025). Furthermore, the confirmed high antioxidant load contributes to the plant's documented anticancer effects (Songserm et al., 2022). Therefore, the reproducible enhancement of TPC and TFC through the polyploidization of *R. nasutus* cell cultures represents a significant step towards developing a more efficient, reliable, and standardized source of raw material for pharmaceutical and health industries (Yaowachai et al., 2025). As well as improving antioxidant activity, elicitation with NaCl and PEG 6000 has also been shown to enhance total phenolic content (TPC) and total flavonoid content (TFC) in various plant species, which are crucial for the plant's therapeutic value (Ahmad et al., 2020). The benefits of elicitation in other plant species have been well-documented, but no reports have investigated the effects of NaCl and PEG 6000 elicitation on callus cultures of *R. nasutus*. This plant has proven medicinal properties, and the stimulation of bioactive compound production in *R. nasutus* callus cultures through NaCl and PEG 6000 elicitation presents an unexplored avenue to enhance its therapeutic potential.

Therefore, the present study aimed to optimize abiotic elicitation using NaCl and PEG 6000 to stimulate the biosynthesis of phenolic and flavonoid compounds in *R. nasutus* callus cultures. It was hypothesized that moderate levels of abiotic stress would enhance total phenolic content, total flavonoid content, and antioxidant activity through reactive oxygen species (ROS)-mediated signaling and activation of defense-related pathways. Conversely, excessive salt or osmotic stress was expected to impair callus growth and pigment stability as a result of cellular dehydration and oxidative damage. The study further anticipated a hormetic response, in which metabolite production increases with mild to moderate stress but declines under severe stress. Understanding these growth–metabolite trade-offs provides an important basis for developing efficient *in vitro* systems for the sustainable production of bioactive metabolites from this medicinal species.

MATERIALS & METHODS

In vitro Callus Induction

Callus cultures of *R. nasutus* were initiated from *in vitro*-derived nodal explants, following a protocol established in a previous study (Yaowachai et al., 2023). A voucher specimen (No. RN-KKU-2025) was deposited in the herbarium of the Department of Biology, Faculty of Science, Naresuan University. The explants were first washed under running tap water for 15min, surface-sterilized with 70% ethanol for 1min, and disinfected using 0.1% (w,v) $HgCl_2$ for 15min. After three rinses with sterile distilled water, the explants were transferred to Murashige and Skoog (MS) medium supplemented with 30g L^{-1} sucrose to promote shoot growth. For surface sterilization of the explants, where $HgCl_2$ was utilized, strict safety protocols were followed: $HgCl_2$ was handled in a chemical fume hood, and all residual solutions and waste were collected and disposed of as regulated hazardous waste according to institutional safety guidelines. Nodal explants were selected due to their higher phenolic content and antioxidant activity compared to leaf explants and derived from a single accession to ensure genetic consistency. The sterile nodes were cut into 0.5cm segments and cultured on MS medium containing 1mg L^{-1} kinetin and 1mg L^{-1} 2,4-D, solidified with 8g L^{-1}, agar and adjusted to pH 5.8 before autoclaving. The cultures were maintained under a 16/8h light/dark cycle at 1,800 lux and 25±2°C for 4 weeks. The illumination level was verified using a calibrated lux meter before incubation to ensure consistent light exposure across all culture shelves.

In vitro Elicitation

Four-week-old cultures of *R. nasutus* calli, previously established on MS medium supplemented with 1mg L^{-1} kinetin and 1mg L^{-1} 2,4-D, were employed for elicitation

experiments. To induce osmotic and drought stress, the calli were transferred to fresh MS media supplemented with varying concentrations of NaCl (0, 50, 100, 150, and 200mM) and PEG 6000 (0, 1, 2, 3, and 4% w,v). The pH values of all the media were adjusted to 5.8 before autoclaving at 121°C for 20min. The calli were incubated under these conditions for 7 days under a 16/8h light/dark photoperiod at 25±2°C with light intensity of 1,800 lux. A 7-day elicitation period was chosen based on preliminary trials showing that *R. nasutus* calli remained viable under stress for up to 7 days, whereas longer exposure resulted in browning and loss of tissue integrity (data not shown). Following elicitation, the treated calli were transferred to elicitor-free MS medium containing the same concentrations of kinetin and 2,4-D for an additional 2-week recovery period under identical environmental conditions. A 2-week recovery phase was provided after elicitation to allow callus tissues to regain metabolic stability and continue secondary metabolite synthesis under normal conditions. This duration was selected based on preliminary observations and is consistent with prior reports where a two-week recovery period was applied to evaluate post-stress adaptation in plants (Batista-Silva et al., 2019). The impacts of the NaCl and PEG 6000 treatments were assessed based on survival rate, biomass accumulation (fresh weight, dry weight, and water content), physiological responses (chlorophyll and carotenoid contents, malondialdehyde levels and electrolyte leakage), and production of bioactive compounds.

Water Content

The water content in *R. nasutus* calli was determined as the difference between the fresh weight (FW) and dry weight (DW) of the samples. FW was measured immediately after harvesting the calli using an analytical balance. To determine DW, the samples were placed in a hot-air oven at 50°C for 5 days or until they reached a constant weight. After drying, the calli were weighed again to obtain the dry weight. The percentage of water in the samples offered insights into their physiological status under different elicitation conditions. Water content was calculated using the formula:

$$\text{Water content (\%)} = [(FW - DW) / FW] \times 100 \quad (1)$$

Electrolyte Leakage

The electrolyte leakage from *R. nasutus* calli was measured to assess membrane stability under different elicitation conditions (Hniličková et al., 2019). A 0.1 g aliquot of fresh callus was weighed and placed in a vial containing 10mL of deionized water. The samples were then incubated in the dark at 25°C for 24h to allow the release of electrolytes. After incubation, the initial electrical conductivity (EC_1) of the solution was measured using a conductivity meter. The samples were then incubated at 100°C for 15min to completely disrupt the cell membranes, allowing maximum release of electrolytes. The samples were then allowed to cool to 25°C, and the final electrical conductivity (EC_2) was measured. The electrolyte leakage was calculated using the formula:

$$\text{Electrolyte leakage (\%)} = (EC_1 / EC_2) \times 100 \quad (2)$$

Malondialdehyde Accumulation

Malondialdehyde (MDA) content, which serves as an indicator of lipid peroxidation, was measured in *R. nasutus* calli using the thiobarbituric acid (TBA) method (Sunohara & Matsumoto, 2004). A 0.2g aliquot of fresh callus was homogenized in 2mL of 0.1% (w,v) trichloroacetic acid (TCA) and centrifuged at 14,000rpm for 5min to separate the debris. The resulting supernatant (1mL) was then mixed with 2mL of 0.5% (w,v) TBA in 20% (w,v) TCA. This mixture was heated at 95°C for 30min to facilitate the formation of the MDA-TBA complex, followed by rapid cooling in an ice bath. After cooling, the mixture was centrifuged at 10,000rpm for 5min to remove any precipitation. The absorbance of the supernatant was then measured at 532nm and 600nm using a UV-Vis spectrophotometer. The MDA content was calculated by subtracting the absorbance at 600 nm from the absorbance at 532nm, with the final result expressed as nmol MDA per gram of fresh weight. This method provided an estimate of oxidative stress in the calli, reflecting the lipid peroxidation level. The MDA content was also calculated using the equation:

$$\text{MDA (nmole g}^{-1}\text{ FW)} = [[(A_{532} - A_{600}) \times V_f \times V_e] / [(155 \times V_a \times FW)]] \times 1000 \quad (3)$$

Where:
V_f = final volume
V_e = volume of TCA used for extraction
V_a = volume of supernatant used for absorbance measurement
F_W = fresh weight of the sample

Chlorophyll and Carotenoid Contents

The chlorophyll and carotenoid contents in *R. nasutus* calli were determined using a modified method based on Dağlioğlu et al. (2022). Briefly, 0.05g of fresh callus tissue was homogenized in 5mL of 80% (v,v) acetone and incubated in darkness at 25°C for 48h. The extract was then centrifuged at 8,000rpm for 10min to separate the solids. The supernatant was collected, and the absorbance was measured at 450, 645, and 663nm using a spectrophotometer. The concentrations of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids were calculated using the formulas:

$$\text{Chlorophyll } a \text{ (mg/g FW)} = [(12.7xA_{663})-(2.69xA_{645})]x[(V/(1000xW)] \quad (4)$$
$$\text{Chlorophyll } b \text{ (mg/g FW)} = [(22.9xA_{645})-(4.68xA_{663})]x[(V/(1000xW)] \quad (5)$$
$$\text{Total chlorophyll (mg/g FW)} = [(20.2xA_{645})+(8.02xA_{663})]x[(V/(1000xW)] \quad (6)$$
$$\text{Carotenoids (mg/g W)} = (4.07xA450)-[(0.0435x\text{Chlorophyll } a)+ (0.3367x\text{Chlorophyll } b)] \quad (7)$$

where:
A = absorbance, V = volume of 80% acetone, W = weight of callus in grams

Crude Extraction

Callus tissues of *R. nasutus* treated with various concentrations of NaCl and PEG 6000 were extracted for phytochemical and antioxidant analyses following the protocol of Yaowachai et al. (2023). The samples were dried in a hot-air oven at 50°C for 7 days to ensure complete dehydration, and then finely ground to a uniform

powder using a laboratory grinder. For extraction, 0.1g of powdered callus was mixed with 1mL of methanol and subjected to ultrasound-assisted extraction (UAE) at 40 kHz and 30°C for 30min. This process was repeated five times to optimize compound recovery. The extracts were centrifuged at 14,000rpm, filtered, and concentrated under reduced pressure at 45°C for 12h. The dried extracts were stored at -20°C for further analyses.

Total Phenolic Content

The total phenolic content (TPC) was quantified using the Folin–Ciocalteu method, with minor modifications from Hmamou et al. (2022). Briefly, 20µL of each methanolic extract (1mg mL^{-1}) was added to a 96-well microplate, followed by 100µL of 10% (v,v) Folin–Ciocalteu reagent and 80µL of 7% (w,v) sodium carbonate (Na_2CO_3). After 30min of dark incubation at room temperature, the absorbance was measured at 760nm. Each sample was assayed in quintuplicate. Gallic acid was used as the standard (10-320µg mL-1, 2-fold serial dilutions), and results were expressed as mg of gallic acid equivalent per gram of crude extract (mg GAE g^{-1} extract). The calibration curve followed the equation $y = 0.0072x + 0.0324$ ($R^2 = 0.9995$).

Total Flavonoid Content

The total flavonoid content (TFC) was measured using the aluminum chloride colorimetric method, modified from Barbouchi et al. (2020). In brief, 10µL of each extract (1mg mL^{-1}) was mixed with 6µL of 5% (w,v) $NaNO_2$ and incubated for 5min. Then, 12µL of 10% (w,v) $AlCl_3$ was added and further incubated for 5min. Next, 60µL of 1 M NaOH and 112µL of deionized water were added to adjust the total volume to 200µL. The absorbance was recorded at 510 nm. The samples were analyzed in quintuplicate. A quercetin standard (20-640µg mL-1) was used to construct the calibration curve ($y = 0.0003x + 0.0021$, $R^2 = 0.9992$), and results were expressed asmg of quercetin equivalent per gram of crude extract (mg QE g^{-1} extract).

Antioxidant Activity Assessment via FRAP Assay

The ferric reducing antioxidant power (FRAP) assay was performed according to Niroula et al. (2021), with slight modifications. The FRAP reagent was freshly prepared by mixing sodium acetate buffer (300mM, pH 3.6), 10mM TPTZ in 40mM HCl, and 20mM $FeCl_3 \cdot 6H_2O$ in a 10:1:1 ratio. After pre-incubation at room temperature for 10min, 10µL of each extract (2mg mL^{-1}) was mixed with 190µL of FRAP reagent and incubated at 37°C for 15min in the dark. The absorbance was measured at 593 nm. Trolox was used as the standard (0.31-320µg mL-1), and the antioxidant capacity was expressed as mg of Trolox equivalent per gram of dry extract (mg TE g^{-1} extract), based on the linear calibration curve ($y = 0.0049x + 0.0033$, $R^2 = 0.9999$). All measurements were conducted in quintuplicate.

Antioxidant Activity Assessment via DPPH Assay

The DPPH radical scavenging assay was conducted according to Li et al. (2021), with minor adjustments. Equal volumes (100µL) of 0.2mM DPPH solution and serially diluted extracts were mixed and incubated in the dark at room temperature for 30min. The absorbance was recorded at 517nm. Ascorbic acid served as the positive control, with methanol as the negative control. The free radical scavenging activity (FRSA) was calculated as:

$$FRSA\ (\%) = [1 - (A_{sample} / A_{control})] \times 100 \quad (8)$$

where A_{sample} represents the absorbance of the extract, and $A_{control}$ corresponds to the absorbance of the negative control. The half-maximal inhibitory concentration (IC_{50}), indicating the concentration required to achieve 50% free radical scavenging activity, was determined by plotting the percentage inhibition against extract concentration.

Antioxidant Activity Assessment via ABTS Assay

The ABTS radical scavenging activity was assessed following Ehiobu et al. (2021), with slight modifications. The $ABTS^{\bullet+}$ radical cation was generated by reacting 7.0mM ABTS with 2.45mM potassium persulfate (1:1, v,v) and incubating the mixture in the dark for 12-16 h. The solution was diluted to an absorbance of 0.70±0.03 at 734 nm. For the assay, 50µL of each diluted extract was mixed with 150µL of $ABTS^{\bullet+}$ solution and incubated in the dark for 10min. The absorbance was measured at 734 nm. The percentage inhibition was calculated as in the DPPH assay, and the IC_{50} values were determined accordingly. Ascorbic acid was used as a reference standard, with all measurements conducted in quintuplicate.

Statistical Analysis

The statistics of this study were based on a Completely Randomized Design (CRD). The fundamental experimental unit was defined as a single callus clump cultured independently in one bottle, resulting in a sample size of n = 10 biological replicates for each treatment. All quantitative results were presented as the mean±SE. Data analysis primarily utilized IBM SPSS Statistics version 22.0, where One-way Analysis of Variance (ANOVA) was employed to assess differences among treatments. Critically, before conducting ANOVA and subsequent post-hoc comparisons, all datasets were checked for the essential parametric assumptions of normality of residuals and homoscedasticity (homogeneity of variances). Duncan's multiple range test was used for post hoc comparisons, with statistical significance determined at $P<0.05$. Finally, Pearson's correlation coefficients were calculated using OriginPro® 2024 to evaluate relationships among the measured variables, and Hierarchical Cluster Analysis (HCA) and heatmap visualization based on the correlation matrices were performed using R Studio to classify sample groups and explore patterns of variation across the treatments.

RESULTS

***In vitro* elicitation**

Elicitation is a widely used strategy in plant tissue culture to enhance stress tolerance and secondary metabolite production. Combinations of NaCl and PEG 6000 as elicitors provide insights into the adaptive responses of plant cells under varying stress conditions.

R. nasutus, a medicinal plant known for its bioactive compounds, serves as an ideal model for studying the effects of osmotic and salinity stress on callus growth. This study investigated the growth parameters and physiological traits of *R. nasutus* calli under different NaCl and PEG 6000 concentrations. The findings provide valuable information on optimal stress conditions that promote biomass accumulation while maintaining callus viability.

The survival rate of *R. nasutus* calli was 100% across most treatments, except for the highest PEG concentration (4%) combined with 200mM NaCl, where the survival rate decreased to 80.00±0.00% (Table 1). Results suggested that extreme osmotic stress conditions negatively impacted callus viability. The effect of PEG 6000 and NaCl on callus induction and morphology was evaluated *in vitro*. The calli exhibited differential responses in terms of texture, growth rate, and color (Fig. 1). At lower concentrations of PEG 6000 (1%) and NaCl (50mM), the calli remained friable with a yellowish-white appearance, indicating active cell division and moderate water stress adaptation. As PEG 6000 and NaCl concentrations increased, a transition from friable to compact calli was observed. At high concentrations of PEG 6000 (3%) and NaCl (200mM), the calli exhibited a compact structure with reduced proliferation and a brownish appearance, indicative of stress-induced senescence (Fig. 1).

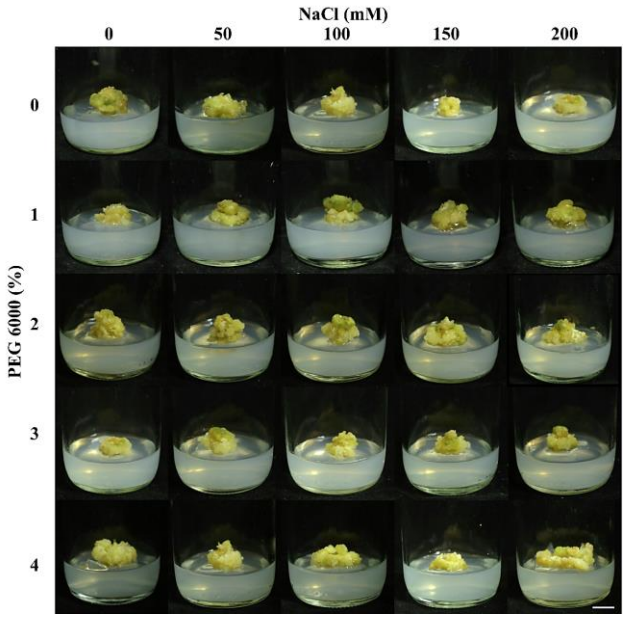

Fig 1: Callus formation under different concentrations of PEG 6000 and NaCl elicitors (scale bar = 1cm).

Fresh weight differed drastically among the treatments ($P<0.05$). The highest fresh weight (1.82±0.21g) was observed in calli treated with 4% PEG and 100mM NaCl, followed by calli exposed to 2% PEG alone (1.58±0.09g). By contrast, the lowest fresh weight was noted in the control group (0.87±0.05g) and in calli subjected to 3% PEG with 200mM NaCl (0.77±0.08g). These results indicated that moderate levels of osmotic stress enhanced callus biomass, whereas excessive stress conditions reduced callus biomass. The dry weight of calli followed a similar trend to fresh weight, with significant alterations between the treatments ($P<0.05$). The highest dry weight (1.26±0.26g) was recorded in calli treated with 100mM NaCl alone, while the lowest (0.07±0.00g) was observed in the control treatment (Table 1).

The elicitation of *R. nasutus* calli with varying concentrations of NaCl and PEG 6000 resulted in significant changes in water content, electrolyte leakage, and malondialdehyde (MDA) levels. The water content of the calli generally decreased with increasing PEG and NaCl concentrations, with the highest reduction observed at 4% PEG combined with 200mM NaCl (87.30±1.09 %, Table 1). Similarly, electrolyte leakage increased with stress, reaching a maximum at 150mM NaCl (85.77±1.04%), indicating higher membrane damage. MDA levels, reflecting oxidative stress, varied significantly across treatments, with the highest levels recorded in calli treated with 3% PEG and 200mM NaCl (90.19±1.08nmol g^{-1} FW, Table 1), suggesting elevated lipid peroxidation under severe stress conditions. PEG and NaCl stress led to significant physiological alterations in *R. nasutus* calli, with a combined impact on membrane integrity and oxidative stress.

The elicitation of *R. nasutus* callus with different NaCl and PEG 6000 concentrations significantly affected chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids. The chlorophyll *a* content showed a decreasing trend with increasing PEG and NaCl concentrations, with the lowest value (0.04±0.00mg g^{-1} FW, Table 2) observed in calli treated with 4% PEG, across all NaCl levels, except for 100mM NaCl. Similarly, chlorophyll *b* content declined under stress, with the lowest value (0.07±0.00mg g^{-1} FW) recorded under several stress conditions, indicating a reduction in the photosynthetic pigment pool. Total chlorophyll content followed a similar trend, dropping to a minimum of 0.11±0.00mg g^{-1} FW and showing significant degradation under osmotic and salt stress (Table 2). Carotenoid contents also exhibited a reduction under stress, with the lowest recorded value of 0.17±0.00mg g^{-1} FW under 2 and 4% PEG with 50mM NaCl treatments. Some treatments showed relatively stable carotenoid levels, possibly indicating a protective role against oxidative stress. These results suggested that NaCl and PEG stress led to a significant decline in photosynthetic pigment content in *R. nasutus* calli, potentially impacting growth and physiological functions.

Phytochemical Contents and Antioxidant Activities

Secondary metabolites production in plants is influenced by environmental stressors like salinity and drought. Elicitation using abiotic stressors as NaCl and PEG 6000 has been widely utilized to enhance the accumulation of bioactive compounds in plant tissue cultures. *R. nasutus*, a medicinal plant established for its antioxidant with pharmacological properties, responds to these stressors by modulating its physiological and biochemical pathways. This study investigated the impact of different concentrations of NaCl and PEG 6000 on the TPC and TFC of *R. nasutus* calli to better understand how stress-induced metabolic changes impacted secondary metabolite production.

Table 1: Survival percentages, fresh weight, dry weight, water content, electrolyte leakage percentage and malondialdehyde contents of *R. nasutus* calli elicited under different concentrations of PEG and NaCl

Elicitors		Survival (%)	Fresh weight (g)	Dry weight (g)	Water content (%)	Electrolyte leakage (%)	Malondialdehyde(nmol g^{-1} FW)
PEG (%)	NaCl (mM)						
0	0	100±0.00^{a}	0.87±0.05^{c-e}	0.07±0.00^{c}	91.31±0.57^{a-c}	69.45±2.48^{d-h}	30.58±2.86^{g-i}
1	0	100±0.00^{a}	0.8±0.07^{d-e}	0.07±0.00^{c}	90.56±0.91^{a-d}	62.46±1.05^{g-j}	19.55±0.89^{l}
2	0	100±0.00^{a}	1.58±0.09^{a-d}	0.14±0.01^{bc}	91.2±0.81^{a-c}	72.72±5.46^{b-g}	24±0.77^{j-l}
3	0	100±0.00^{a}	1.26±0.26^{a-e}	0.11±0.02^{bc}	91.25±0.69^{a-c}	56.78±4.88^{ij}	21.1±0.57^{l}
4	0	100±0.00^{a}	1.13±0.25^{a-e}	0.13±0.03^{bc}	88.19±0.18^{b-d}	52.84±1.71^{j}	29.61±0.24^{hi}
0	50	100±0.00^{a}	1.04±0.13^{a-e}	0.08±0.01^{bc}	92.02±0.46^{ab}	66.76±4.44^{f-i}	32.32±1.67^{f-h}
1	50	100±0.00^{a}	0.81±0.22^{d-e}	0.08±0.02^{bc}	90.31±0.87^{a-d}	70.82±6.75^{c-h}	22.45±2.15^{kl}
2	50	100±0.00^{a}	1.57±0.26^{a-d}	0.13±0.01^{bc}	90.97±0.97^{a-c}	83.16±7.54^{ab}	37.16±1.13^{de}
3	50	100±0.00^{a}	1.46±0.25^{a-e}	0.11±0.02^{bc}	92.81±0.37^{a}	59.8±5.68^{h-j}	37.35±0.84^{de}
4	50	100±0.00^{a}	1.67±0.15^{ab}	0.17±0.01^{a-c}	89.78±0.42^{a-d}	68.98±2.71^{e-h}	32.13±0.64^{f-h}
0	100	100±0.00^{a}	1.7±0.37^{ab}	0.26±0.13^{a}	86.74±4.69^{d}	79.69±2.86^{a-e}	27.48±2.32 $^{h-j}$
1	100	100±0.00^{a}	1.69±0.34^{ab}	0.17±0.03^{a-c}	89.67±0.64^{a-d}	83.5±1.04^{ab}	27.48±1.83^{h-j}
2	100	100±0.00^{a}	1.7±0.24^{ab}	0.14±0.02^{bc}	91.91±0.40^{ab}	81.71±1.95^{a-c}	42.78±0.77^{c}
3	100	100±0.00^{a}	1.11±0.16^{a-e}	0.09±0.01^{bc}	91.83±0.49^{ab}	69.03±3.13^{e-h}	35.42±0.66^{e-g}
4	100	100±0.00^{a}	1.82±0.21^{a}	0.16±0.01^{a-c}	90.84±0.58^{a-d}	70.75±2.20^{c-h}	31.16±0.56^{f-i}
0	150	100±0.00^{a}	1.1±0.22^{a-e}	0.1±0.02^{bc}	90.85±0.35^{a-d}	85.77±1.04^{a}	41.03±0.49^{cd}
1	150	100±0.00^{a}	1.61±0.35^{a-c}	0.16±0.03^{a-c}	90.26±1.09^{a-d}	70.65±2.80^{c-h}	27.29±1.45^{h-j}
2	150	100±0.00^{a}	1.69±0.36^{ab}	0.16±0.03^{a-c}	90.44±0.72^{a-d}	76.29±2.14^{a-f}	63.68±0.77^{b}
3	150	100±0.00^{a}	0.82±0.12^{d-e}	0.09±0.02^{bc}	88.97±0.86^{a-d}	69.98±3.10^{d-h}	38.32±0.79^{c-e}
4	150	100±0.00^{a}	1.18±0.13^{a-e}	0.15±0.01^{bc}	87.45±0.54^{cd}	73.85±1.26^{b-f}	35.81±1.62^{ef}
0	200	100±0.00^{a}	0.98±0.12^{b-e}	0.11±0.01^{bc}	88.27±0.59^{b-d}	80.63±0.50^{a-d}	30.58±2.34^{g-i}
1	200	100±0.00^{a}	1.71±0.23^{ab}	0.2±0.03^{ab}	88.4±1.05^{b-d}	83.48±1.19^{ab}	26.71±2.15^{i-k}
2	200	100±0.00^{a}	1.2±0.27^{a-e}	0.14±0.02^{bc}	87.61±1.08^{cd}	79.08±1.19^{a-e}	39.87±0.36^{c-e}
3	200	100±0.00^{a}	0.77±0.08^{e}	0.09±0.01^{bc}	88.46±1.37^{b-d}	77.12±2.56^{a-f}	90.19±1.08^{a}
4	200	80±0.00^{b}	1.19±0.29^{a-e}	0.14±0.03^{bc}	87.3±1.09^{cd}	75.98±1.03^{a-f}	37.36±3.88^{de}

Mean±SE values followed by different superscripts in the same column are significantly different according to Duncan's multiple range test (P<0.05).

Table 2: Chlorophyll *a*, chlorophyll *b*, total chlorophyll and carotenoid contents of *R. nasutus* calli elicited under different concentrations of PEG and NaCl

Elicitors		Chlorophyll *a* (mg g^{-1} FW)	Chlorophyll *b* (mg g^{-1} FW)	Total chlorophyll (mg g^{-1} FW)	Carotenoids (mg g^{-1} FW)
PEG (%)	NaCl (mM)				
0	0	0.08±0.00^{a}	0.09±0.00^{ab}	0.17±0.00^{a}	0.33±0.03^{a}
1	0	0.06±0.00^{b-d}	0.08±0.00^{b-d}	0.14±0.00^{bc}	0.27±0.01^{bc}
2	0	0.05±0.00^{e-i}	0.07±0.00^{c-f}	0.12±0.00^{d-i}	0.23±0.01^{c-f}
3	0	0.04±0.00^{j}	0.07±0.00^{c-f}	0.11±0.00^{f-i}	0.18±0.00^{ij}
4	0	0.04±0.00^{f-i}	0.07±0.00^{d-f}	0.11±0.00^{f-i}	0.18±0.01^{ij}
0	50	0.04±0.00^{g-i}	0.08±0.00^{b-e}	0.12±0.00^{c-h}	0.21±0.02^{d-j}
1	50	0.05±0.00^{b-f}	0.09±0.00^{ab}	0.14±0.01^{bc}	0.24±0.01^{c-e}
2	50	0.04±0.00^{j}	0.06±0.00^{f}	0.1±0.00^{i}	0.17±0.00^{ij}
3	50	0.04±0.00^{f-i}	0.07±0.00^{d-f}	0.12±0.01^{e-i}	0.19±0.01^{g-j}
4	50	0.04±0.00^{g-i}	0.07±0.00^{ef}	0.11±0.00^{g-i}	0.17±0.01^{j}
0	100	0.06±0.00^{b-e}	0.09±0.00^{a-c}	0.14±0.00^{b-d}	0.25±0.01^{cd}
1	100	0.05±0.00^{e-i}	0.08±0.00^{c-f}	0.12±0.01^{c-h}	0.2±0.01^{e-j}
2	100	0.05±0.00^{c-g}	0.08±0.00^{b-d}	0.13±0.01^{c-g}	0.24±0.02^{c-e}
3	100	0.04±0.00^{j}	0.07±0.00^{d-f}	0.11±0.00^{g-i}	0.18±0.00^{h-j}
4	100	0.05±0.00^{e-i}	0.07±0.00^{d-f}	0.12±0.00^{e-i}	0.19±0.01^{f-j}
0	150	0.06±0.00^{b}	0.09±0.00^{a-c}	0.16±0.00^{ab}	0.3±0.02^{ab}
1	150	0.05±0.00^{c-h}	0.08±0.00^{b-d}	0.13±0.00^{c-f}	0.25±0.01^{cd}
2	150	0.05±0.00^{e-i}	0.08±0.01^{c-f}	0.12±0.01^{c-h}	0.2±0.01^{f-j}
3	150	0.05±0.01^{d-h}	0.08±0.01^{c-e}	0.13±0.02^{c-h}	0.23±0.03^{c-g}
4	150	0.04±0.00^{ji}	0.07±0.00^{ef}	0.11±0.00^{hi}	0.2±0.01^{f-j}
0	200	0.05±0.00^{e-i}	0.08±0.01^{b-e}	0.13±0.01^{c-h}	0.22±0.01^{c-i}
1	200	0.05±0.00^{c-h}	0.09±0.00^{a-c}	0.14±0.01^{b-e}	0.23±0.01^{c-f}
2	200	0.05±0.00^{d-h}	0.07±0.01^{c-f}	0.12±0.01^{c-h}	0.22±0.02^{c-h}
3	200	0.06±0.00^{bc}	0.1±0.01^{a}	0.16±0.01^{ab}	0.25±0.02^{cd}
4	200	0.04±0.00^{f-i}	0.07±0.00^{d-f}	0.12±0.00^{e-i}	0.22±0.01^{d-i}

Mean±SE values followed by different superscripts in the same column are significantly different according to Duncan's multiple range test (P<0.05).

Results revealed a significant variation in TPC and TFC under different elicitor treatments. The highest TPC value (26.96±0.69mg GAE g^{-1} extract, Table 3) was observed in calli treated with 1% PEG alone, suggesting that moderate salt stress enhanced phenolic compound accumulation. As the PEG concentration increased, a gradual decline in TPC was observed, with the lowest recorded value at 4% PEG and 50mM NaCl (12.15±0.50mg GAE g^{-1} extract), indicating the inhibitory effect of severe osmotic stress. Similarly, TFC showed a decreasing trend with higher PEG concentrations, with the highest value (17.08 ±0.86mg QE g^{-1} extract) at 0% PEG and 100mM NaCl, while the lowest (3.42±0.81mg QE g^{-1} extract) was recorded in calli subjected to 3% PEG and 100mM NaCl (Table 3). These findings suggested that moderate salt stress promoted secondary metabolite biosynthesis, whereas excessive osmotic stress, induced by PEG, suppressed phenolic and flavonoid accumulation.

In plant tissue cultures, abiotic stress is essential for regulating the synthesis of secondary metabolites and antioxidant capacity. The FRAP, DPPH, and ABTS assays were used to evaluate changes in the antioxidant ability of *R. nasutus* calli under different elicitation conditions. Results indicated significant variations in antioxidant activity across treatments. The FRAP assay gave the highest antioxidant activity (33.40±0.53mg TE g^{-1} extract, Table 3)

Table 3: Antioxidant activities by FRAP, DPPH and ABTS assays of *R. nasutus* calli elicited under different concentrations of PEG and NaCl

Elicitor		FRAP assay	IC_{50} by DPPH assay	IC_{50} by ABTS assay	TPC	TFC
PEG (%)	NaCl (mM)	(mg TE g^{-1} extract)	(mg mL^{-1})	(mg mL^{-1})	(mg GAE g^{-1} extract)	(mg QE g^{-1} extract)
0	0	13.68±0.47^{i}	0.59±0.00^{l-n}	0.22±0.00^{jk}	20.93±0.25^{c}	13.25±0.26^{cd}
1	0	26.87±0.62^{b}	0.46±0.00^{pq}	0.16±0.00^{p}	26.49±1.31^{a}	13.92±0.72^{bc}
2	0	6.42±0.21^{l}	1.26±0.00^{ef}	0.31±0.00^{d}	16.28±0.52^{d-f}	8.42±0.31^{hi}
3	0	4.7±0.19^{m}	2.45±0.09^{a}	0.4±0.00^{a}	15.1±0.75^{fg}	4.41±0.56^{lm}
4	0	15.11±0.23^{h}	0.92±0.00^{h}	0.23±0.00^{j}	12.76±0.45^{gh}	6.58±0.26^{i-k}
0	50	18.32±0.47^{ef}	0.59±0.00^{lm}	0.2±0.00^{m}	23.53±0.23^{b}	14.08±0.70^{bc}
1	50	19.48±0.75^{e}	0.59±0.00^{l-n}	0.17±0.00^{op}	21.75±0.22^{bc}	16.75±0.72^{a}
2	50	6.77±0.15^{l}	1.43±0.01^{d}	0.3±0.00^{ef}	17.94±0.39^{de}	12.42±0.87^{c-e}
3	50	6.26±0.23^{l}	2.26±0.09^{b}	0.36±0.01^{b}	14.57±0.63^{f-h}	9.92±0.91^{f-h}
4	50	11.67±0.45^{j}	1.31±0.01^{e}	0.29±0.00^{g}	12.15±0.50^{h}	5.59±1.13^{j-l}
0	100	18.23±0.97^{ef}	0.71±0.00^{jk}	0.22±0.01^{k}	26.38±0.51^{a}	17.08±0.86^{a}
1	100	33.4±0.53^{a}	0.49±0.00^{o-q}	0.15±0.00^{q}	22.87±0.97^{bc}	15.58±0.31^{ab}
2	100	6.19±0.15^{l}	1.52±0.01^{c}	0.34±0.01^{c}	14.86±0.65^{fg}	4.58±0.21^{lm}
3	100	9.8±0.21^{k}	1.54±0.03^{c}	0.32±0.00^{d}	14.16±1.71^{f-h}	3.42±0.81^{lm}
4	100	11.79±0.27^{j}	1.16±0.01^{g}	0.28±0.01^{h}	15.21±0.62^{fg}	5.08±0.31^{k-m}
0	150	23.81±0.86^{c}	0.55±0.01^{m-o}	0.18±0.00^{no}	26.96±0.69^{a}	13.25±0.59^{cd}
1	150	23.07±0.47^{c}	0.41±0.00^{q}	0.14±0.00^{r}	22.25±0.60^{bc}	13.75±0.62^{b-d}
2	150	7.52±0.07^{l}	1.58±0.00^{c}	0.31±0.00^{de}	15.14±0.23^{fg}	6.75±0.49^{i-k}
3	150	12.48±0.29^{ij}	0.84±0.01^{i}	0.25±0.00^{j}	16.75±0.62^{d-f}	4.58±0.43^{lm}
4	150	23.58±0.61^{c}	0.64±0.00^{kl}	0.18±0.01^{n}	18.54±0.02^{d}	10.58±0.55^{e-g}
0	200	21.19±0.67^{d}	0.53±0.00^{m-p}	0.17±0.00^{p}	22.67±0.56^{bc}	11.75±0.61^{d-f}
1	200	21.7±0.30^{d}	0.5±0.00^{n-p}	0.17±0.00^{p}	23.07±0.93^{bc}	12.42±0.53^{c-e}
2	200	9.5±0.47^{k}	1.2±0.03^{fg}	0.3±0.00^{fg}	15.67±2.14^{ef}	7.25±0.67^{ij}
3	200	16.58±0.36^{g}	1±0.01^{h}	0.21±0.00^{l}	17.74±0.35^{de}	9.42±0.43^{gh}
4	200	17.09±0.26^{fg}	0.78±0.00^{ij}	0.18±0.00^{n}	14.99±0.52^{fg}	10.25±0.33^{f-h}

Mean±SE values followed by different superscripts in the same column are significantly different according to Duncan's multiple range test ($P<0.05$).

in calli treated with 1% PEG and 100mM NaCl, suggesting that moderate salt stress enhanced the antioxidant potential. Increasing PEG concentration generally resulted in a decline in FRAP values, with the lowest activity (4.70±0.19mg TE g^{-1} extract) recorded in 3% PEG without NaCl treatment (Table 3). The lowest IC_{50} value measured by the DPPH assay, indicating the highest free radical scavenging activity, was recorded in calli treated with 1% PEG and 150mM NaCl (0.41±0.00mg.mL^{-1}), whereas higher PEG concentrations increased the IC_{50} values, indicating reduced antioxidant capacity. Similarly, the ABTS assay revealed that calli treated with 1% PEG and 150mM NaCl exhibited the highest antioxidant activity, with the lowest IC_{50} value (0.14±0.00mg.mL^{-1}), while a decline was observed at higher PEG levels (Table 3). These findings suggested that moderate salt stress enhanced the antioxidant defense mechanisms in *R. nasutus* calli, while severe osmotic stress induced by PEG 6000 negatively affected plant antioxidant potential.

Pearson's Correlation

The elicitation of *R. nasutus* calli using NaCl and PEG 6000 induced significant physiological and biochemical responses, as reflected in the Pearson's correlation analysis of various physiological and biochemical traits (Fig. 2). Fresh weight showed a strong positive correlation with dry weight (r = 0.70, $P<0.001$), indicating that biomass accumulation was consistent across treatments. Water content showed a significant negative correlation with DW (r = -0.58, $P<0.001$), suggesting that stress conditions reduced water retention in calli tissues. Electrolyte leakage demonstrated a weak but significant positive correlation with malondialdehyde (r = 0.22, $P<0.05$), confirming its role as an indicator of membrane damage under stress. Photosynthetic pigments with chlorophyll *a*, chlorophyll *b*, and total chlorophyll exhibited great positive correlations (r = 0.78, $P<0.001$), indicating co-regulation in response to stress conditions.

TPC and TFC showed a strong positive correlation (r = 0.77, $P<0.001$, Fig. 2), highlighting the co-accumulation of phenolic and flavonoid compounds during stress adaptation. When antioxidant activity was expressed as % inhibition (IC_{50}), strong positive correlations were observed between TPC, TFC, and the antioxidant assays (DPPH, ABTS, and FRAP), particularly between DPPH and ABTS (r = 0.94, $P<0.001$). This positive association confirms that higher phenolic content contributed to greater antioxidant capacity. The FRAP assay also correlated positively with both TPC and TFC, supporting the involvement of phenolic antioxidants in redox homeostasis. These findings provide critical insights into the physiological and biochemical adaptations of *R. nasutus* calli to osmotic and salt stress, emphasizing the interplay between oxidative stress markers, antioxidant defense, and secondary metabolite accumulation.

Hierarchical Cluster Analysis

Hierarchical cluster analysis (HCA) provided an in-depth understanding of the effects of NaCl and PEG 6000 elicitation on the growth and physiological traits of *R. nasutus* calli. The heatmap visualization revealed distinct clustering patterns, indicating differential responses of the calli to varying stress conditions. Callus samples were grouped into distinct clusters based on their physiological and biochemical characteristics, reflecting variations in stress adaptation. Electrolyte leakage and malondialdehyde, both indicators of oxidative stress and membrane damage, exhibited strong variations across samples, with some treatments showing significantly elevated levels (represented by intense red regions). By contrast, traits associated with photosynthetic pigment content with chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids formed a separate cluster with lower values in samples exposed to higher stress levels,

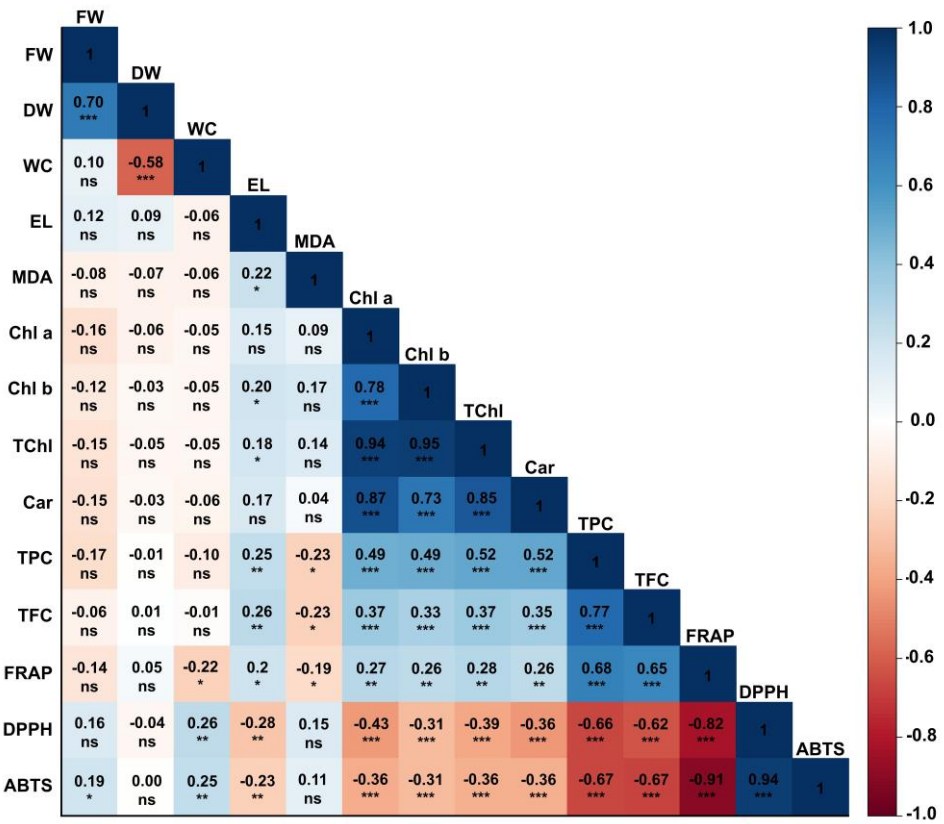

Fig. 2: Pearson's correlation coefficient matrix between growth parameters, physiological traits, phenolic and flavonoid contents and antioxidant activities of *R. nasutus* calli (significance level: * P<0.05, ** P<0.01, ***P<0.001).

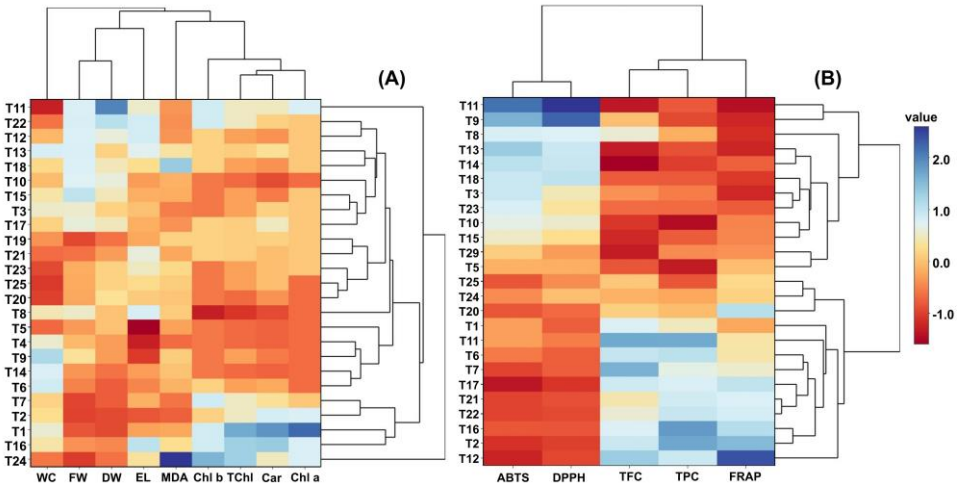

Fig. 3: Hierarchical cluster analysis (HCA) and heatmap of *R. nasutus* calli under different treatments. (A) Physiological traits, (B) TPC, TFC, and antioxidant activities.

as indicated by the blue shading (Fig. 3A). Fresh weight and dry weight clustered together, suggesting that biomass accumulation was similarly influenced by stress conditions. Water content was positioned in a distinct cluster, highlighting its independent response to NaCl and PEG 6000 treatments. These clustering patterns emphasized the differential regulation of growth and physiological traits under salt and osmotic stress conditions, demonstrating the complex interplay between oxidative stress markers, pigment degradation, and biomass accumulation. The observed clustering suggested that NaCl and PEG 6000 elicitors induced varying degrees of physiological stress, ultimately influencing the metabolic status and adaptive mechanisms of *R. nasutus* calli.

HCA was performed to assess the biochemical responses of *R. nasutus* calli to NaCl and PEG 6000 elicitation, focusing on antioxidant activity and secondary metabolite accumulation. Antioxidant assays including ABTS, DPPH, and FRAP exhibited a clear separation from TPC and TFC, suggesting differential regulatory mechanisms (Fig. 3B). Lower ABTS and DPPH values, indicating higher antioxidant activity, were observed in specific treatments (represented by blue-shaded regions), while higher values (red regions) corresponded to lower antioxidant capacity. Interestingly, TPC and TFC exhibited an opposite pattern, clustering separately with predominantly higher values in samples with reduced antioxidant activity, and suggesting a complex relationship between phenolic accumulation and radical scavenging efficiency under stress conditions. The FRAP assay demonstrated variability across treatments, clustering distinctly from DPPH and ABTS, indicating differences in antioxidant response mechanisms. These clustering patterns emphasized the metabolic shifts induced by NaCl

and PEG 6000, illustrating the complex interplay between stress-induced oxidative damage, antioxidant defense, and secondary metabolite production in *R. nasutus* calli. The observed biochemical differentiation further supported the role of osmotic and salt stress in modulating metabolic pathways, potentially influencing the adaptive responses of the plant.

DISCUSSION

In vitro Elicitation

Elicitation is a well-established strategy in plant tissue culture to increase stress tolerance and stimulate secondary metabolites production. Current study investigated the effects of combined osmotic and salinity stress, induced by NaCl and PEG 6000, on growth, physiological characteristics including pigment contents of *R. nasutus* calli. Our findings provided valuable insights into the adaptive responses of plant cells under varying stress conditions, and highlighted the potential of elicitation to optimize biomass accumulation while maintaining callus viability. The experimental findings indicated that the survival rate of *R. nasutus* calli was unaffected under most treatments, with a 100% survival rate, except under the highest stress condition of 4% PEG combined with 200mM NaCl, where survival decreased to 80%. This suggested that extreme osmotic stress negatively impacted callus viability, likely due to excessive dehydration and impaired cellular function (Qi et al., 2023). Variations in the fresh and dry weight examined in *R. nasutus* calli demonstrated the influence of osmotic and salinity stress on biomass accumulation. In our study, the highest fresh weight was obtained under moderate stress (4% PEG and 100mM NaCl), whereas the lowest values were recorded under the most severe stress conditions. Similarly, dry weight decreased significantly at higher stress levels, with the highest values found in calli exposed to 100mM NaCl. These results concurred with previous reports showing that moderate osmotic stress promoted biomass accumulation, whereas excessive stress suppressed growth by disrupting cellular homeostasis (Song et al., 2017). Similarly, Lin et al. (2022) demonstrated that PEG-induced osmotic stress effectively simulated drought conditions and led to marked reductions in both fresh and dry biomass. Their findings further revealed that increasing PEG concentrations restricted water uptake, thereby impairing growth and decreasing water content (fresh weight) and structural biomass (dry weight).

The physiological response of *R. nasutus* calli to NaCl and PEG 6000 stress was impacted by water content, electrolyte leakage, malondialdehyde (MDA) levels, chlorophyll, and carotenoid contents. Water content decreased significantly with increasing stress levels, with the most pronounced reduction observed under 4% PEG and 200mM NaCl. This decline in water retention suggested that osmotic stress induced dehydration, a common response to high salinity and drought conditions (Askari-Khorasgani et al., 2021). Electrolyte leakage, an indicator of membrane integrity, increased with stress severity, peaking at 85.77±1.04% under 150mM NaCl. The MDA content, one important measure of the lipid peroxidation and oxidative membrane damage, was highest in callus treated with 3% PEG and 200mM NaCl. Results suggested that combined osmotic and salt stress enhanced the reactive oxygen species (ROS) production, thereby aggravating oxidative damage to cellular membranes (Skrzydlewska et al., 2005).

Comparable discoveries were investigated by Pradhan et al. (2021), who demonstrated that PEG-induced osmotic stress in mango calli led to a pronounced increase in MDA levels, particularly in more susceptible monoembryonic genotypes such as *Dashehari and Amrapali*. This response reflected enhanced lipid peroxidation and greater oxidative damage under drought conditions. In line with these results, the elevated MDA content observed in *R. nasutus* calli under severe NaCl and PEG stress in our study indicated that prolonged osmotic and ionic stress promoted excessive ROS production, aggravating membrane lipid peroxidation, and impaired cellular integrity (Miller et al., 2010). Electrolyte leakage provided additional evidence of membrane damage and oxidative injury. In tomato (*Solanum lycopersicon* L.), Aazami et al. (2021) reported that both drought and salinity stresses significantly increased electrolyte leakage, reflecting enhanced membrane permeability and oxidative damage. This finding concurred with our results under NaCl and PEG treatments, confirming that elevated electrolyte leakage values can be considered a reliable indicator of stress-induced membrane destabilization and ROS accumulation.

The impact of NaCl and PEG stress on photosynthetic pigment content was also significant, as chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids decreased with increasing stress levels (Sadak et al., 2024). Chlorophyll *a* and *b* contents exhibited the lowest values under 4% PEG treatment, demonstrating substantial degradation of the photosynthetic apparatus. Total chlorophyll followed a similar trend, reaching its lowest concentration under 4% PEG, further confirming the opposing impacts of osmotic and salinity stress on chlorophyll stability and photosynthetic efficiency (Akhter et al., 2021). Carotenoid levels, which help prevent oxidative damage, were also reduced, with the lowest recorded value under 4% PEG and 50mM NaCl. However, some treatments maintained relatively stable carotenoid levels, suggesting a potential protective role in mitigating oxidative stress (Lim et al., 2023). Similar observations were proved by Suminar et al. (2022), who studied the effects of PEG-induced drought stress on iaa9 tomato mutants. Their findings confirmed that chlorophyll content was highly sensitive to increased PEG concentrations, with chlorophyll *b* and total chlorophyll showing reductions of up to 44 and 23% under 15% PEG, respectively. The progressive decline in chlorophyll under increasing PEG concentration reflected disruption of electron transport and photochemical activity in photosystem II, leading to photodamage and elevated ROS formation (Fan et al., 2017). The reduction in photosynthetic pigments under stress conditions indicated a decline in photosynthetic efficiency, possibly impairing callus growth and

physiological functions (Rodrigues et al., 2024). These findings highlighted that NaCl and PEG stress significantly impaired pigment synthesis, ultimately affecting photosynthesis and growth in *R. nasutus* calli.

Our results demonstrated that moderate levels of osmotic and salinity stress enhanced biomass accumulation in *R. nasutus* calli, while severe stress led to cellular damage, oxidative stress, and reduced growth. These results provide important new information about how plant cells adapt to stress and highlight the potential of elicitation schemes to optimize secondary metabolite generation in medicinal plants. Future investigation should focus on identifying molecular aspects underlying these stress responses and exploring the potential of stress-induced elicitation to improve the bioactive compounds production in *R. nasutus* and other medicinal species.

Phytochemical Contents and Antioxidant Activities

This study investigated the impact of NaCl and PEG 6000 elicitation on TPC, TFC, and antioxidant activities of *R. nasutus* callus cultures. Our results demonstrated that abiotic stress, specifically moderate salinity, significantly influenced the accumulation of secondary metabolites and enhanced the antioxidant potential in *R. nasutus*. However, excessive osmotic stress induced by high concentrations of PEG 6000 had an inhibitory effect on these parameters. Results indicated that moderate salt stress (150mM NaCl) enhanced TPC and TFC in *R. nasutus* calli, with the highest TPC observed at 0% PEG and 150mM NaCl. This result aligned with previous studies, showing that moderate salinity stimulated phenolic compounds production as part of the defensive mechanism against oxidative stress (Bistgani et al., 2019). It is commonly known that phenolic compounds, including flavonoids, act as antioxidants and protect plants from ROS generated under stressful conditions (Hajam et al., 2023). However, the decline in TPC and TFC under higher PEG concentrations (e.g., 4% PEG and 50mM NaCl) suggested that severe osmotic stress inhibited secondary metabolite accumulation. This result was consistent with findings that excessive osmotic stress disrupted cellular homeostasis, leading to reduced metabolic activity and secondary metabolite production (Mashabela et al., 2023). The pattern observed in *R. nasutus* calli concurred with findings in white poplar (*Populus alba*) clones subjected to PEG-induced osmotic stress, where increasing PEG concentrations led to reductions in biomass and photosynthetic pigments while simultaneously elevating TPC, TFC, proline, glycine betaine, and antioxidant activity (Vuksanović et al., 2023). The observed decrease in TFC under higher PEG concentrations (e.g., 3% PEG and 100mM NaCl) further supported the notion that severe osmotic stress negatively impacted flavonoid biosynthesis. Flavonoids are produced throughout the phenylpropanoid pathway, which is sensitive to environmental stressors (Agati et al., 2012). The suppression of this pathway under severe osmotic stress may explain the reduced TFC values. Our findings highlighted the importance of optimizing stress conditions to maximize secondary metabolite generation under *in vitro* cultures.

The present study demonstrated that moderate salinity (150mM NaCl) significantly enhanced TPC, TFC, and antioxidant activity in *R. nasutus* callus cultures, whereas high concentrations of PEG 6000 exerted an inhibitory effect. These results are in line with earlier findings proving that osmotic or salt-induced stress can act as an elicitor to stimulate secondary metabolite accumulation as part of the plant defense mechanism against oxidative stress. For instance, Sharma et al. (2023) reported that moderate NaCl stress in *Withania somnifera* microshoots enhanced withaferin A production without markedly affecting growth, highlighting the role of controlled osmotic stress in promoting secondary metabolism. Similarly, PEG treatment in *Catharanthus roseus* callus cultures enhanced anticancer alkaloid production, although the effect was concentration-dependent, with higher PEG levels potentially limiting metabolite accumulation (Abdulwahid et al., 2024). In *Ocimum basilicum*, moderate PEG concentrations promoted callus survival and morphogenic growth, whereas excessive osmotic stress disrupted cellular homeostasis and reduced metabolic activity (Osman et al., 2020). This highlights the importance of optimizing elicitor concentrations, such as NaCl and PEG, to maximize secondary metabolite production and antioxidant capacity under plant tissue culture system.

The antioxidant activity of *R. nasutus* calli, as evaluated by the FRAP, DPPH, and ABTS assays, showed significant variations under different stress conditions. The highest antioxidant activity was observed in calli treated with 1% PEG and 100mM NaCl, suggesting that moderate salt stress enhanced plant antioxidant defense mechanisms, consistent with studies showing that moderate stress upregulated antioxidant enzymes and increased the production of non-enzymatic antioxidants, such as phenolics and flavonoids (Gill & Tuteja, 2010). The decline in antioxidant activity under higher PEG concentrations (e.g., 3% PEG without NaCl) further supported the detrimental effects of severe osmotic stress on antioxidant potential. The DPPH and ABTS assays revealed that calli treated with 1% PEG and 150mM NaCl exhibited the highest free radical scavenging activity, as indicated by the lowest IC_{50} values. These results suggested that moderate salt stress enhanced the production of antioxidant compounds, which are crucial for mitigating oxidative damage caused by ROS (Kesawat et al., 2023). Both assays are based on the ability of antioxidants to quench stable radicals: ABTS generates a pre-formed radical cation ($ABTS^{\bullet+}$) that reacts with hydrogen or electron-donating compounds, while DPPH interacts with antioxidants, the purple DPPH• radical is reduced to its non-radical state (Wołosiak et al., 2021). However, potential overestimation of antioxidant capacity can occur due to the interactions of some antioxidants with substrates or intermediate products. Compared with ascorbic acid, a standard antioxidant, *R. nasutus* callus extracts showed lower radical scavenging activity. The antioxidant defense system plays a vital role in helping plants cope with stressful conditions by slowing down conditioned cell death. While plants lack sufficient antioxidant enzymes to neutralize redundant ROS, cellular organelles fail to maintain normal functions,

leading to lipid peroxidation, oxidative damage to proteins, DNA and nucleic acid degradation, and inhibition of various enzymes (Fujita & Hasanuzzaman, 2022). Despite this, the elicited calli demonstrated considerable antioxidant potential, especially under moderate stress conditions, highlighting abiotic stress role in stimulating bioactive antioxidant compounds production.

The important improvement of antioxidant capacity, characterized with the highest FRAP assay besides the lowest inhibitory concentration values for DPPH and ABTS (IC_{50}), under the condition of moderate combined stress (e.g., 1% PEG + 100-150mM NaCl), represents a clear hormetic response in *R. nasutus* calli. This phenomenon is a critical adaptive mechanism where non-lethal stress levels induce a defensive reaction, primarily mediated by cellular redox signaling (Ahmed et al., 2021). Moderate abiotic stress, such as the combined osmotic and salinity stress, triggers the controlled generation of ROS, particularly hydrogen peroxide, which acts not merely as a toxic agent but as a crucial signaling molecule that coordinates the cell's adaptive defense response (Sana et al., 2025). This H_2O_2-mediated cascade is known to activate various transcription factors like WRKY and MYB that regulate downstream defense genes (Qahtan et al., 2022), effectively redirecting metabolic flux toward antioxidant secondary metabolites biosynthesis (Akula & Ravishankar, 2011). This upregulation targets key enzymes in the flavonoid and phenylpropanoid pathways. The committed step, catalyzed by Phenylalanine Ammonia-Lyase (PAL), is strongly activated via abiotic stressors like salinity, which shunts L-phenylalanine into the production of phenolics (Chen et al., 2024). Further downstream, enzymes like Chalcone Synthase (CHS), the entry point into the flavonoid branch, and subsequently Dihydroflavonol 4-Reductase (DFR), are upregulated to facilitate the high accumulation of TPC and TFC, which directly correlate with the observed superior FRAP and radical scavenging activities (Ahmed et al., 2021). This adaptive increase in antioxidant compounds serves to mitigate potential oxidative damage.

In contrast, severe stress levels exceed the cell's capacity for controlled signaling, leading to overwhelming oxidative stress, loss of membrane integrity evidenced by increased electrolyte leakage and lipid peroxidation products like malondialdehyde and ultimate cellular damage, resulting in suppressed metabolite synthesis and reduced viability (Punetha et al., 2022). From a production standpoint, while the callus system in this study effectively demonstrates the optimization of elicitation, it is important to note that callus extracts often contain lower concentrations of bioactive compounds compared to differentiated plant parts or more specialized *in vitro* cultures (Qahtan et al., 2022). For potential industrial scale-up, systems like hairy root cultures are generally more advantageous, exhibiting rapid growth, genetic stability, and significantly higher accumulation of certain secondary metabolites (e.g., tanshinone, vilforin) due to their differentiated root morphology and high biosynthetic capacity (Asyakina et al., 2021; Hussain et al., 2022). Similarly, optimized cell suspension cultures can offer superior biomass accumulation and better scalability for the production of specific compounds, such as rosmarinic acid in other species like *Satureja khuzistanica*, where production levels were reported to achieve up to 4,350mg L^{-1} under optimized culture conditions with phenylalanine and sucrose (Sahraroo et al., 2016). Therefore, future work should compare the elicited callus with these alternative culture systems to maximize the commercial yield of *R. nasutus* bioactive compounds.

Our findings show low-to-moderate stress levels stimulating bioactivity, followed by high-dose inhibition, characterize a classic biphasic dose-response, or hormesis (Calabrese, 2014). At low-to-moderate concentrations (e.g.,1% PEG and 100-150mM NaCl), the mild stress triggers an adaptive compensatory response, indicating to an expressive increase in secondary metabolite production (TPC and TFC) without severe inhibition of growth. In contrast, high concentrations of PEG or NaCl induce a toxic effect, causing a sharp decline in both growth and bioactivity, which corresponds to the inhibitory phase of the hormetic curve (Habibi et al., 2025). This dual response stimulation at low doses and inhibition at high doses is recognized as an adaptive physiological strategy in plants to cope with adverse environments (Godínez-Mendoza et al., 2023).

To comprehensively address the limitations of the current study, future research must incorporate advanced analytical and molecular techniques, such as relying solely on bulk quantification methods like TPC and TFC fails to identify the specific bioactive compounds. The use of Ultra-High-Performance Liquid Chromatography coupled with Mass Spectrometry (UHPLC-MS/MS) is essential for achieving detailed metabolite profiling and chemotyping to precisely separate, identify, and quantify individual phenolic and flavonoid compounds that contribute to the observed antioxidant activity (Bajkacz et al., 2018; Dzakovich et al., 2025). Furthermore, a complete understanding of the biosynthetic mechanism requires molecular validation via Quantitative Real-Time PCR (qPCR) (Gao et al., 2024). This technique should target important genes in the phenylpropanoid pathway with PAL (phenylalanine ammonia-lyase), CHS (chalcone synthase), F3H (flavanone 3-hydroxylase), and DFR (dihydroflavonol 4-reductase) to determine the transcriptional regulation underlying secondary metabolite accumulation in response to NaCl and PEG stress (Aluko et al., 2025; Pineda-Hidalgo et al., 2025). By integrating these sophisticated chemical and molecular analyses, researchers can establish a clear relationship between the abiotic stress, gene expression profiles, and the production of specific bioactive compounds in *R. nasutus* callus cultures (Jia et al., 2022).

The use of NaCl and PEG 6000 as elicitors provided a practical approach to enhancing bioactive compound production in plant tissue cultures, with significant meaning for the pharmaceutical and nutraceutical industries (Khyahrii et al., 2025). Our results demonstrated that moderate salt stress enhanced the phytochemical content and antioxidant activity of *R. nasutus* calli, while severe osmotic stress had inhibitory effects. These results focus on the significance of optimizing stress conditions to

maximize secondary metabolite production and antioxidant potential in medicinal plants. Future research should explore the molecular mechanisms underlying stress-induced metabolic changes and investigate the application of other abiotic and biotic elicitors to further enhance bioactive compound production in plant tissue cultures.

Conclusion

This study demonstrates that abiotic elicitation using NaCl and PEG 6000 significantly influences the growth, physiology, and secondary metabolite accumulation in *R. nasutus* callus cultures. Moderate salinity stress, particularly treatments combining 1% PEG with 100–150mM NaCl, enhanced biomass accumulation, increased phenolic and flavonoid contents, and promoted antioxidant activity. In contrast, severe osmotic stress induced by high PEG concentrations negatively affected callus viability, pigment content, and antioxidant potential, indicating cellular damage and oxidative stress. These findings highlight the importance of optimizing stress levels to stimulate secondary metabolite biosynthesis while maintaining cellular stability. The results provide valuable insights into the application of controlled abiotic stress as a practical strategy to enhance phytochemical production in *R. nasutus* callus cultures, supporting its potential use in pharmaceutical and nutraceutical industries.

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