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Serotonin-Salt-Stress Model-Induced Cell Growth *via* Promoting an Antioxidant System and Secondary Metabolites in *Capsicum annuum* Cell Suspension Culture

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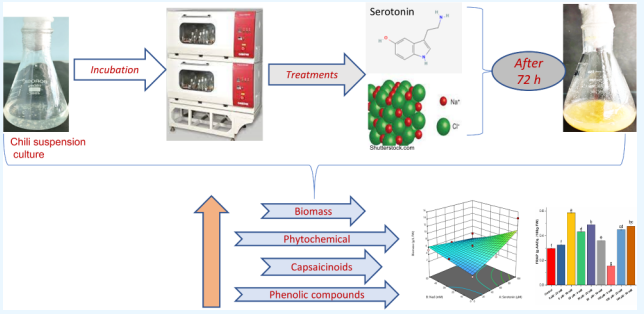
ABSTRACT: *Capsicum annuum* contains potential therapeutic capsaicinoids, and various stress factors influence plant productivity. Serotonin is an indoleamine involved in signaling several stress response mechanisms in plants. However, the influence of serotonin on cell growth and the accumulation of secondary metabolites, mainly capsaicinoids production, is not yet clearly defined under salt stress. In this study, we optimized chili cell suspension cultures to maximize biomass, capsaicinoids, and phenolic compounds production using response surface methodology with two variables (serotonin and NaCl) of different concentrations in culture media supplemented with 2,4-dichlorophenoxyacetic acid and Kinetin. The results revealed a significant increase in biomass (14.3 g/L FW), capsaicin (0.93 μg/g FW), and dihydrocapsaicin content (0.32 μg/g FW) in chili cell suspension cultures compared with the control. Among all the phenolic compounds, chlorogenic acid was enhanced (17.4 μg/g FW), compared to control cultures. Serotonin exhibited stress mitigation effects and boosted antioxidant potential in chili suspension cultures. The present results illustrated that the optimized conditions can be used in scale-up studies of capsaicinoids production through the bioreactor.



1. INTRODUCTION

Plants confront several biotic and abiotic environmental stress factors during their life cycles. Among abiotic stresses, salinity is one of the ecological parameters that negatively affects the growth of plant and crop productivity.[1] Plant survival depends on activating efficient protective responses during salinity stress. Osmotic equilibrium is disturbed, leading to the generation of reactive oxygen species in plants, the primary source of cascade reactions during all the biotic and abiotic stress.[2] Salinity stress disrupts cellular ion homeostasis, uptake of nutrient systems, activation of enzymes, synthesis of natural compounds, antioxidant properties of the hormones, and modulation of hormones, which reduce biomass production and cell death.[2] The disruption in the equilibrium stimulates secondary metabolite pathways for various defense mechanisms.[3] The plant secondary metabolites production is induced by several biotic or abiotic stress factors involved in metabolic signaling pathways, which affect plant cellular and molecular processes.[4]

Chili (*Capsicum annuum*) is one of the essential commercially important crops, and it is widely used as a nutritional, economic, industrial, and medicinal plant worldwide.[5−7] It belongs to the Solanaceae family. It contains essential nutrients (vitamins A, C, and E) and secondary metabolites such as phenolics, flavonoids, carotenoids, and alkaloids, which play important roles in human health.[8−10] Several environmental factors, including salinity stress, have an indispensable effect on chili growth and its production; previous studies documented the impact of salinity stress on callus growth and capsaicinoids production.[5] Therefore, to cope with stress and maintain cellular homeostasis, there is a need to find an efficient growth regulator to increase plant tolerance, growth, and yield of the chili plant for its survival.[5,7] Recently, the application of serotonin has been proven to be an effective method to increase plant tolerance against salinity stress in plants.[11]

Serotonin is a tryptophan-based indole molecule synthesized during the melatonin biosynthesis pathway and ubiquitously found across all living forms on Earth.[12] Serotonin exhibits diverse plant roles, including morphogenesis, growth regulation, ripening, stress tolerance, and defense mechanism.[1,11,12] Structurally similar to melatonin, it acts as a signaling molecule that regulates various biotic and abiotic stresses by modulating secondary metabolite pathways.[13] According to previous studies, exogenous serotonin treatment induces growth and

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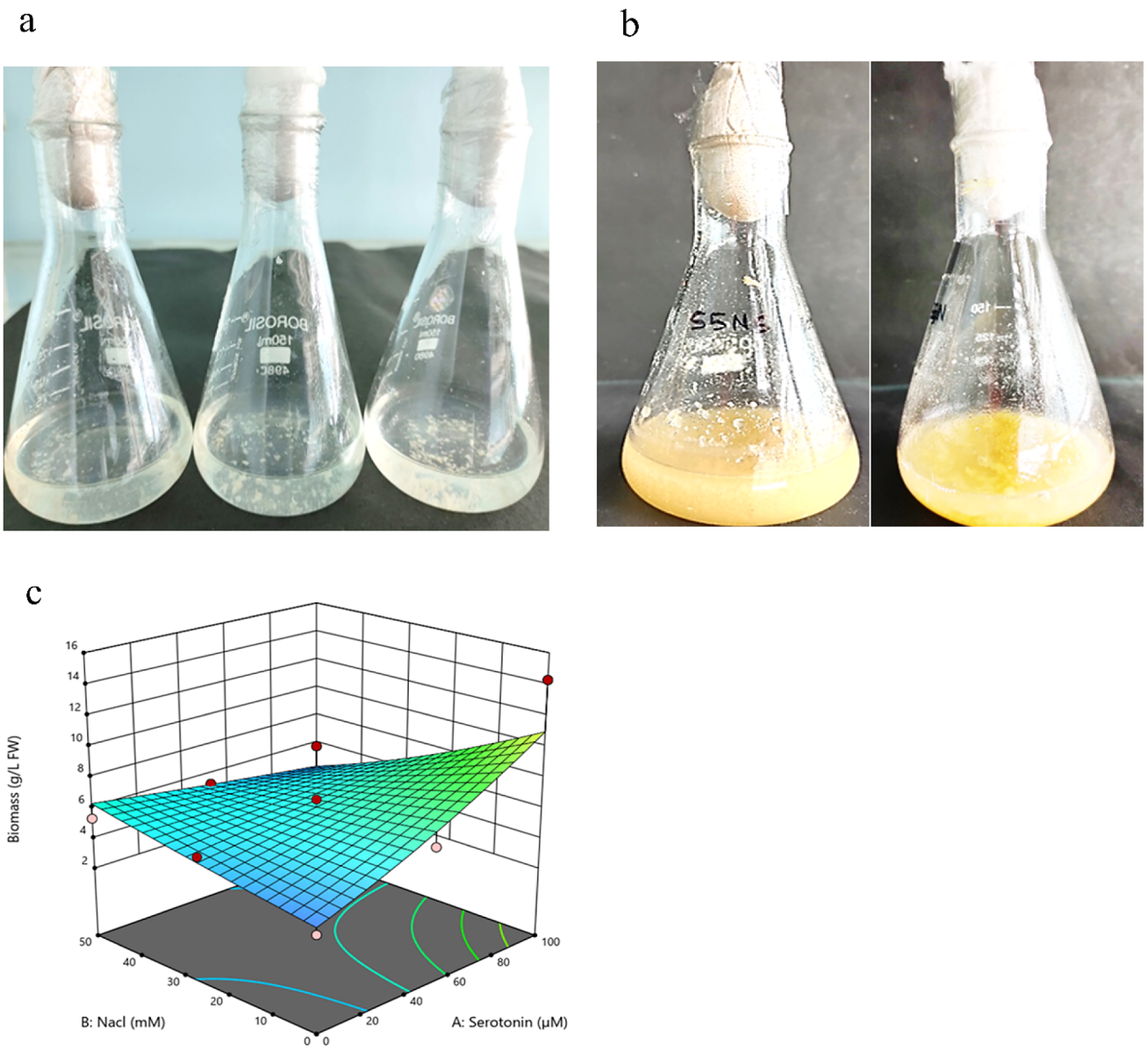

Figure 1. (a) Chili cell suspension cultures in the flask after inoculation in MS medium supplemented with 2, 4-D (2.0 mg L^{-1}), and Kin (0.5 mg L^{-1}) media. (b) Chili cell suspension cultures were grown in a flask after serotonin and NaCl treatment. (c) 3D response surface plot for evaluating the interactive effects of two independent variables (serotonin and NaCl) on the cell biomass in the chili cell suspension cultures.

stress tolerance in rapeseed and tomato seedlings under salt stress.[14,15] However, endogenous serotonin levels respond to thermal stress in *Hypericum perforatum*[16] and insect pest infestation in rice.[17] Furthermore, the genes associated with the serotonin biosynthetic pathway were strongly up-regulated in soybean, Arabidopsis, tomato, rice, and sorghum under heat, salt, and drought stress.[1,18] In addition, the exogenous application of serotonin and melatonin enhanced the biomass and isoflavone production and triggered its biosynthetic pathway genes in soybean under temperature stress.[11,12]

Several studies have elucidated the role and various functions of serotonin in several plant species under salinity stress. However, there is no research on the effects of serotonin and NaCl by employing response surface methodology on cell growth and secondary metabolite production in chili cell suspension cultures. Improving the accumulation and production of secondary metabolites in chili, such as capsaicinoids, phenolics, and flavonoids, is of interest to many researchers due to their pharmacological application. Capsaicinoids are important alkaloids produced by the phenylpropanoid and branch-chain fatty acid pathways. The most abundant capsaicinoids are capsaicin and dihydrocapsaicin, responsible for about 90% of the pungency in chili.[19] The bioactive compounds in chili, such as capsaicinoids and phenolic compounds, have several applications in the food and pharmaceutical industries.[20] Therefore, there is a great demand for producing valuable secondary metabolites in *in vitro* callus cultures under controlled biotic and abiotic elicitors.[21] Conventional methods for improving and producing specific metabolites, such as capsaicinoids and phenolic compounds, by one variable at a time is an expensive and time-consuming process.[22]

The response surface methodology is a simple, precise, and statistical method that optimizes the operational conditions to understand the primary parameters to enhance capsaicinoids without affecting the cost of production.[23,24] This method

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Table 1. Central Composite Design for the Minimum and Maximum Coded Levels of Serotonin and NaCl

Factor	Minimum	Maximum	Coded Low	Coded High
A - Serotonin	0.000	100.00	-1 ↔ 0.00	+1 ↔ 100.00
B - NaCl	0.000	50.00	-1 ↔ 0.00	+1 ↔ 50.00

focuses on multiple variables with a minimum number of experimental trials with valid statistical results to construct an empirical model.[22] In previous studies, many researchers aimed to enhance the capsaicinoids content using different RSM extraction methods. No research was documented on serotonin's role in capsaicinoids and phenolic compound production in chili suspension cultures. Thus, this is the first study to assess the influence of serotonin and NaCl on biomass, capsaicinoids, phenolic compounds production, and their antioxidant potential in chili suspension cultures. This will provide us with the optimized conditions for the *in vitro* production of capsaicinoids and phenolic compounds in chili suspension cultures to be used as ingredients in the pharmaceutical, food, and cosmetic industry.

2. MATERIALS AND METHODS

2.1. Chemicals. All HPLC standards, serotonin (SER), capsaicin, dihydrocapsaicin, protocatechuic acid, catechin, caffeic acid, rutin, p-coumaric acid, ferulic acid, quercetin, chlorogenic acid, and trans-cinnamic acid, were procured from Sigma-Aldrich, Bengaluru, India. All HPLC-grade solvents, chemicals, and reagents, including NaCl, plant growth regulators, and DPPH, were purchased from Hi-media, Mumbai, India.

2.2. Callus Induction and Chili Cell Culture Establishment. Chili (*Capsicum annuum*) seeds, Meghana variety, were procured from the ICAR-Indian Institute of Horticultural Research, Bangalore, India. Fresh and healthy matured green chili fruits were collected from a greenhouse established in the Plant Cell Biotechnology Department, CSIR, Central Food Technological Research Institute, Mysore. The fruits were surface sterilized,[25] and the chili placenta was inoculated on MS medium (Murashige and Skoog 1962) containing 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2, 4-D) and 0.5 mg L^{-1} kinetin (Kin) supplemented with 3% (w/v) sucrose along with 0.3% (w/v) phytagel (Sigma-Aldrich). The pH of the medium was adjusted to 5.8 before autoclaving. The chili cultures were incubated at an intensity of 45 μmol m^{-2} s^{-1}, 24 °C under 16 h light/8 h dark photoperiod, and further induced friable callus was subcultured every month to initiate suspension cultures. The inoculum for suspension culture was 12 g L^{-1} inoculated into a 150 mL Erlenmeyer flask comprising 40 mL of liquid MS medium supplemented with 2, 4-D (2.0 mg L^{-1}) and Kin (0.5 mg L^{-1}) shown in Figure 1a. Chili cell suspension cultures were incubated at 24 °C on an orbital shaker (ORBITEK Scigenics Biotech, India) at 90 rpm under a photoperiod.[19]

2.3. Experimental Design using a Response Surface Methodology. One of the most suitable tools for the assessment, analysis, and modeling various operating variables resulting from interactions is the response surface methodology. Based on our earlier published work, the current study used Design Expert software version 8.0 (Stat Ease Inc. trial version) to determine the optimal levels of serotonin and NaCl constituents.[11] The experimental design employed two independent variables, including (*A*) serotonin and (*B*) NaCl (Table 1). Each variable at different concentrations optimized cell growth and secondary metabolite production in chili cell suspension cultures. After 30 days of the growth phase, according to the obtained RSM experimental design Table 2,

Table 2. Central Composite Design for the Optimization of Serotonin and NaCl

std	run	factor 1 (serotonin μM)	factor 2 (NaCl mM)
5	1	0	25
6	2	100	25
9	3	50	25
2	4	100	0
10	5	50	50
7	6	50	0
3	7	0	50
4	8	100	50
8	9	0	0

serotonin and NaCl treatment was administered to the grown chili cell suspension cultures. To optimize the concentrations and effects on biomass, total phenolic content, total flavonoid content, antioxidant capacity, capsaicinoids, and phenolic compounds were studied. In this RSM investigation, the experiments were accomplished using the central composite design (CCD) to explain the interaction of process variables and the function of quadratic polynomials. The complete experimental design was performed in a randomized manner and comprised 9 combinations and runs with replicates each at a central point. The data were analyzed by a multiple regression equation to fit in a second-order polynomial model, and model eq 1 is given below:

$$Y = \beta 0 + \sum_{i=0}^{n} \beta iXi + \sum_{i=0}^{n} \beta iiXi^2 + \sum_{i\neq i=1}^{n} \beta ijXiXj \quad (1)$$

where Y is the response variable, β_0 is the intercept obtained during replicated experiments of CCD, n is the number of factors analyzed, β_i, βii, and βij are the linear (cross-product effect), quadratic, and interactive model coefficients, respectively. Accordingly, Xi and Xj indicate the levels of the independently coded variables in the experimental design. In the present work, RSM-CCD was applied to specify the optimum levels, and 3D response surface and contour plots were constructed using design-expert software based on response analysis.

2.4. Measurement of Callus Growth. The chili cell suspension cultures were treated with serotonin and NaCl after the exponential phase (30 days) and were harvested after 72 h of treatment. The cells were harvested and filtered through a

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vacuum filter to separate media. The callus was washed with distilled water to remove any residual media, and the fresh weight (FW) was recorded and expressed in g/L FW.[11]

2.5. Measurement of Total Phenolic Content (TPC). The total phenolic content of chili cell suspension cultures was estimated spectrophotometrically using the Folin-Ciocalteu method.[26] In brief, 0.1 mL of suspension culture extract was taken for the analysis, and sample absorbance was measured at 650 nm. The total phenolic content was determined using an equation obtained from the reference standard gallic acid and expressed as gallic acid equivalent (GA Eq) g/100 g fresh weight.

2.6. Measurement of Total Flavonoid Content (TFC). The total flavonoid content was determined using the aluminum chloride in chili suspension cultures extract.[27] The amount of flavonoid was calculated using a quercetin-derived standard curve as a reference. TFC was measured at 430 nm, using a UV spectrophotometer (Genesis, Thermofisher) and expressed as quercetin equivalent (Qeq) g/100 g fresh weight.

2.7. Antioxidant Assays. *2.7.1. DPPH Radical Scavenging Assay.* The DPPH assay was performed using the method described by Kumar et al. (2020) with slight modifications.[28] Different concentrations of chili suspension culture extract were pipetted out for further analysis, and the reaction mixture was incubated in the dark for 15 min at room temperature. The absorbance of the reaction mixture was measured at 517 nm using a Genesis UV−vis spectrophotometer. The percentage of DPPH radical scavenging activity was calculated by using the following formula:

$$\text{DPPH scavenging activity} = (\text{OD control} - \text{OD sample})/\text{OD control} \times 100$$

2.7.2. Ferric Reducing Antioxidant Power (FRAP) Assay. The FRAP assay was used to evaluate the antioxidant capacity of the samples, following the Oyaizu method.[29] The absorbance of the reaction mixture was measured by using a Genesis UV−vis spectrophotometer at 700 nm. The FRAP antioxidant was calculated based on the change in absorbance and expressed as g/100 g of FW using the standard curve established for ascorbic acid (AA Eq).

2.7.3. Total Antioxidant Activity. The total antioxidant activity (TAA) of the suspension cultures extract was assessed according to the method described by Prieto et al. (1999) to evaluate the antioxidant capacity of the chili suspension cultures extract.[30] The TAC absorbance was recorded at 695 nm and calculated using the obtained equation from the ascorbic acid standard expressed as g/100 g of FW AA Eq.

2.8. Extraction and Quantification of Capsaicinoids by HPLC. Capsaicinoids were extracted from the suspension cultures by following the protocol of González-Zamora et al. (2015).[31] The protocol was slightly modified: One gram of chili suspension culture cells was extracted using 5 mL of acetonitrile, and the samples were stored at −20 °C until analysis. High-Performance Liquid Chromatography (HPLC) was used to quantify the capsaicinoid content using a C18 column (YMC S-5 μm, 250 × 4.6 mm). The mobile phase consisted of water (A) and acetonitrile (B) at a ratio of (40:60, v/v). A 1 mL/min flow rate was used in isocratic mode, and the sample injection volume was 20 μL detected at 280 nm.[6] Capsaicinoid were quantified based on retention time and area obtained from capsaicin and dihydrocapsaicin standard curves. HPLC analysis was conducted in triplicate to ensure linearity and reproducibility.

2.9. Quantification of Phenolic Compounds by HPLC. The identification and quantification of individual phenolic and flavonoids were evaluated using a reported method.[32] The phenolic compounds were detected at 280 and 320 nm using reference standards such as protocatechuic acid, catechin, caffeic acid, rutin, p-coumaric acid, ferulic acid, quercetin, chlorogenic acid, and trans-cinnamic acid. The quantification of each compound was based on the retention time, and by calculating the standard area attained from the HPLC data, calibration curves for standards were created with linearity ($r >$ 0.979−0.991). The standard was injected in triplicate to measure the linearity.

2.9.1. Statistical Analysis. All experiments were conducted in triplicate and expressed as mean ± standard deviation of three replicates. Experimental design, data analysis, and 3D response surface plots were prepared using Design-Expert 11 software (Stat-Ease, Inc., Minneapolis, MN, ABD). Second-order coefficients were generated by regression analysis, whereas the goodness of fit of the models was evaluated by the coefficient of determination (R^2) and ANOVA at the 95% significance level. A second-order model fits the data according to model eq 1 in the experimental design.

3. RESULTS

3.1. Effects of Independent Variables on Cell Growth Employing RSM-CCD. The different combinations of serotonin-NaCl exhibited a significant increase in cell growth, except for higher concentrations of serotonin and NaCl (100 μM:50 mM) as shown in Figure 1b, indicating a negative effect of increased NaCl concentration on cell growth. Cell biomass was significantly elevated at higher serotonin concentrations compared with the control (Figure 1c).

3.2. Effects of Serotonin and NaCl Factors on Total Phenolics and Total Flavonoid Content. The total phenolic and flavonoid content of chili suspension cultures was investigated after 72 h of serotonin-NaCl treatment (Figure 2). A significant increase was observed in the total phenolic content following serotonin-NaCl treatment compared with the control. It was found that among all the combinations of serotonin-NaCl, the maximum total phenolic content (0.74 g/100 g FW) was attained in 50 μM:50 mM serotonin-NaCl compared with control (Figure 2a). Similarly, it is observed that a moderate level of serotonin and a lower amount of NaCl treatment significantly elevated flavonoid content in chili suspension cultures. Compared with the control, the maximum total flavonoid content (0.69 g/100 g FW) was achieved by 50 μM:25 mM serotonin-NaCl treatment (Figure 2b).

3.3. Capsaicinoids Profile Under Serotonin and NaCl Factors. The influence of various combinations of serotonin and NaCl on the different forms of capsaicinoids, *viz.*, capsaicin and dihydrocapsaicin, was investigated. The serotonin and NaCl interaction significantly stimulated the accumulation and production of capsaicin and dihydrocapsaicin in treated cells compared with the control in chili suspension cultures (Figure 3). The outcome of this treatment indicates that the capsaicinoids production is a concentration-dependent response, showing both increment and reduction of capsaicin and dihydrocapsaicin content. Among all the combinations of serotonin-NaCl treatments, the maximum capsaicin content was found (0.93 μg/g FW) in 100 μM:25 mM, respectively,

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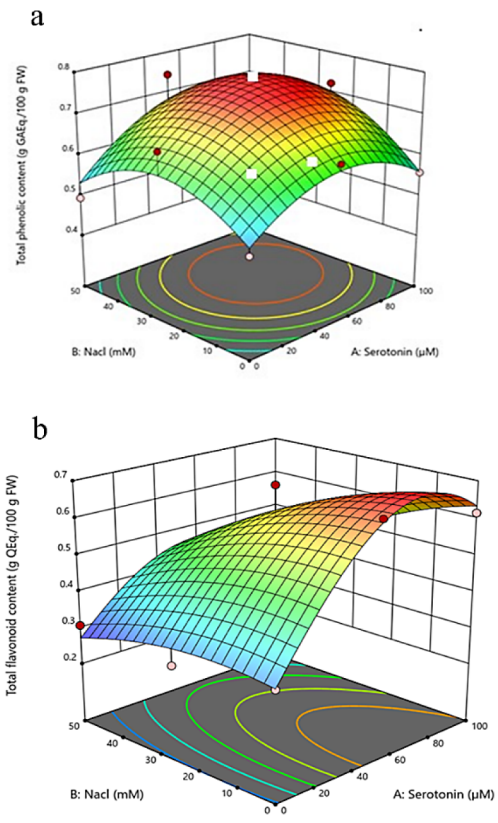

Figure 2. 3D response surface plots for the evaluation of interactive effects of two independent variables (serotonin and NaCl) on the (a) total phenolic content and (b) total flavonoid content in chili cell suspension cultures.

compared with the control, whereas dihydrocapsaicin content showed a significant increase in the lower serotonin and NaCl combination (0.32 μg/g FW) in 50 μM:25 mM, as displayed in Figure 3a and b. Serotonin, individually or in combination with NaCl, positively affected capsaicin production. However, dihydrocapsaicin content was enhanced by all combinations of serotonin-NaCl treatments except NaCl without serotonin and serotonin without NaCl. The best conditions among all treatments for capsaicinoids production were the application of a higher concentration of serotonin and a lower concentration of NaCl.

3.4. Phenolic Profiling Under Serotonin and NaCl Factors. The current study quantified nine phenolic compounds in chili cell suspension cultures by HPLC under serotonin and NaCl treatment. The main phenolic compounds were chlorogenic acid, catechin, caffeic acid, rutin, p-coumaric acid, ferulic acid, quercetin, trans-cinnamic acid, and protocatechuic acid (Figures 4 and 5). The results revealed that the varied combination of serotonin-NaCl significantly increased chlorogenic acid, protocatechuic acid, catechin, caffeic acid, rutin, p-coumaric acid, ferulic acid, quercetin, and trans-cinnamic acid in suspension cultures. Compared with the control, chlorogenic acid was significantly elevated by 18.96-fold in 100 μM serotonin and 25 mM NaCl (17.4 μg/g FW) among all the phenolic compounds (Figure 4a). Likewise, catechin and caffeic acid also showed significant increases in 100 μM serotonin and 25 mM NaCl (20.56 and 6.02 μg/g FW), respectively, compared to control (Figure 4b,c). Moreover, rutin showed a significant increase in serotonin-100 μM without NaCl, which indicates that NaCl did not play

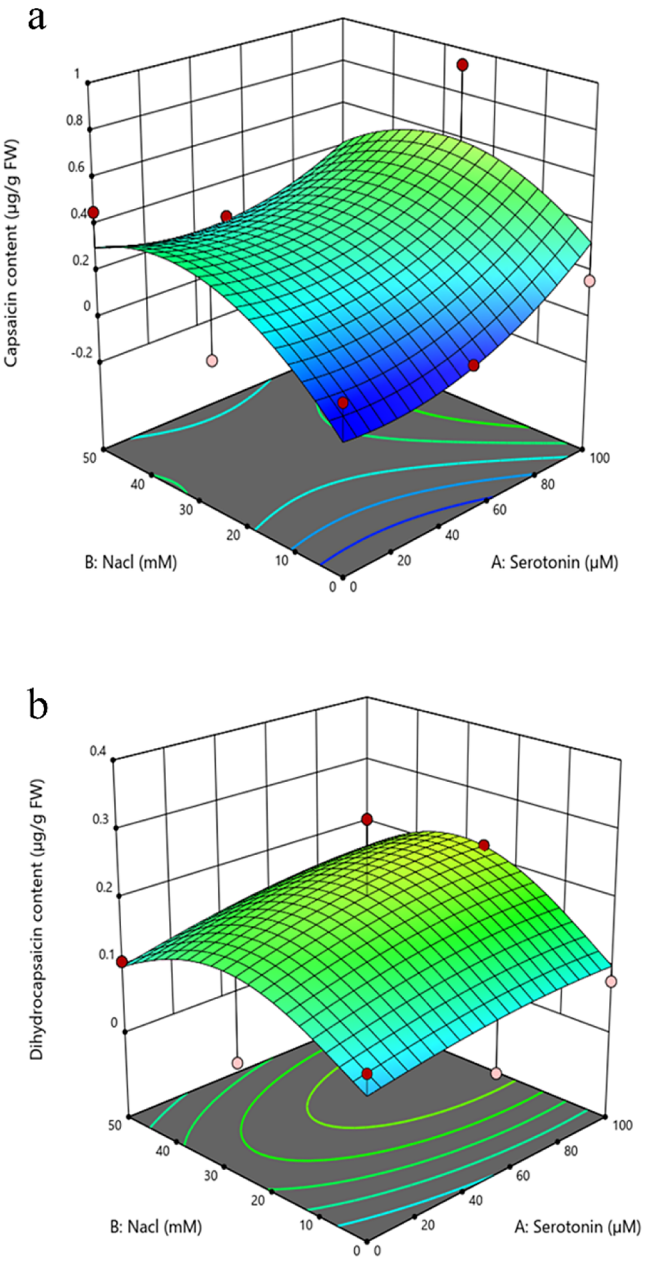

Figure 3. 3D response surface plots for the evaluation of interactive effects of two independent variables (serotonin and NaCl) on the (a) capsaicin and (b) dihydrocapsaicin contents in chili cell suspension cultures.

any vital role in the accumulation of rutin (Figure 4d). The results showed a significant increase in p-coumaric acid and ferulic acid at higher NaCl concentrations than in the control (Figure 4e and f). Subsequently, quercetin, trans-cinnamic acid, and protocatechuic acid contents were also increased in suspension cultures under higher concentrations of serotonin-NaCl treatment compared with the control (Figure 5a–c). As a result, a higher amount of serotonin and NaCl is reported to promote phenolic production in suspension cultures.

3.5. Effects of Serotonin and NaCl Factors on Antioxidant Capacity. The antioxidant capacity of serotonin-NaCl-treated samples was determined using three methods (DPPH, FRAP and TAA). Our study investigated that the DPPH radical scavenging activity showed the highest activity (170 μg/mL) under serotonin-NaCl (100 μM:0 mM) shown

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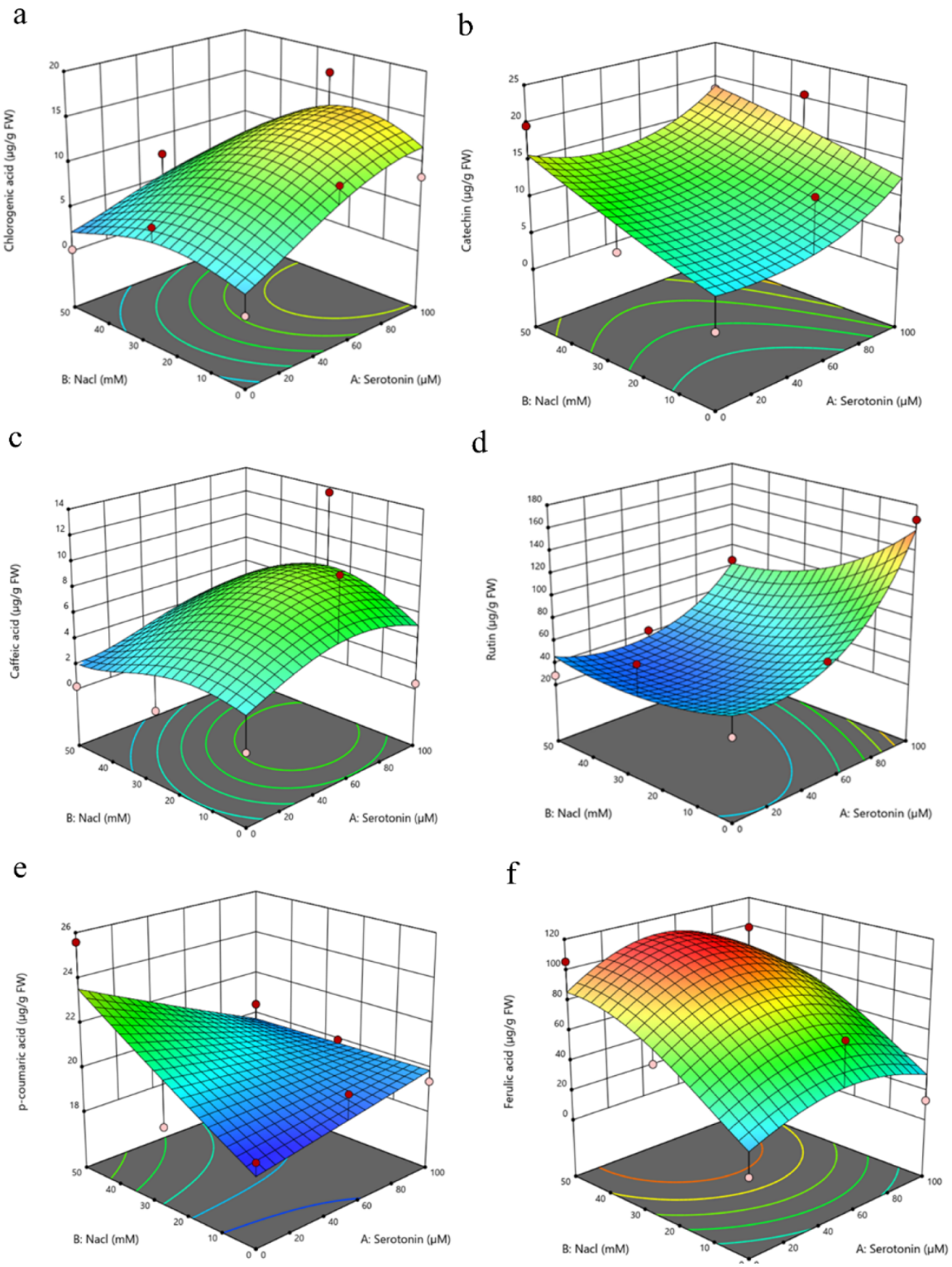

Figure 4. 3D response surface plots for the evaluation of interactive effects of two independent variables (serotonin and NaCl) on the (a) chlorogenic acid, (b) catechin, (c) caffeic acid, (d) rutin, (e) p-coumaric acid, and (f) ferulic acid in chili cell suspension cultures.

in (Figure 6a). Whereas, the FRAP assay showed higher potential in higher concentrations of NaCl without serotonin (0 μM:50 mM) and a moderate level of serotonin and lower level of NaCl (50 μM: 25 mM) was also beneficial to enhance the antioxidant potential in suspension cultures, compared to control (Figure 6b). The total antioxidant activity of chili suspension cultures was significantly increased under nine combinations of serotonin-NaCl treatments (Figure 6c). Higher antioxidant potential was acquired under serotonin-NaCl (100 μM:50 mM) treatment in chili suspension cultures. Notably, higher serotonin concentrations promote cell growth and antioxidant capacity in suspension cultures better than in the control.

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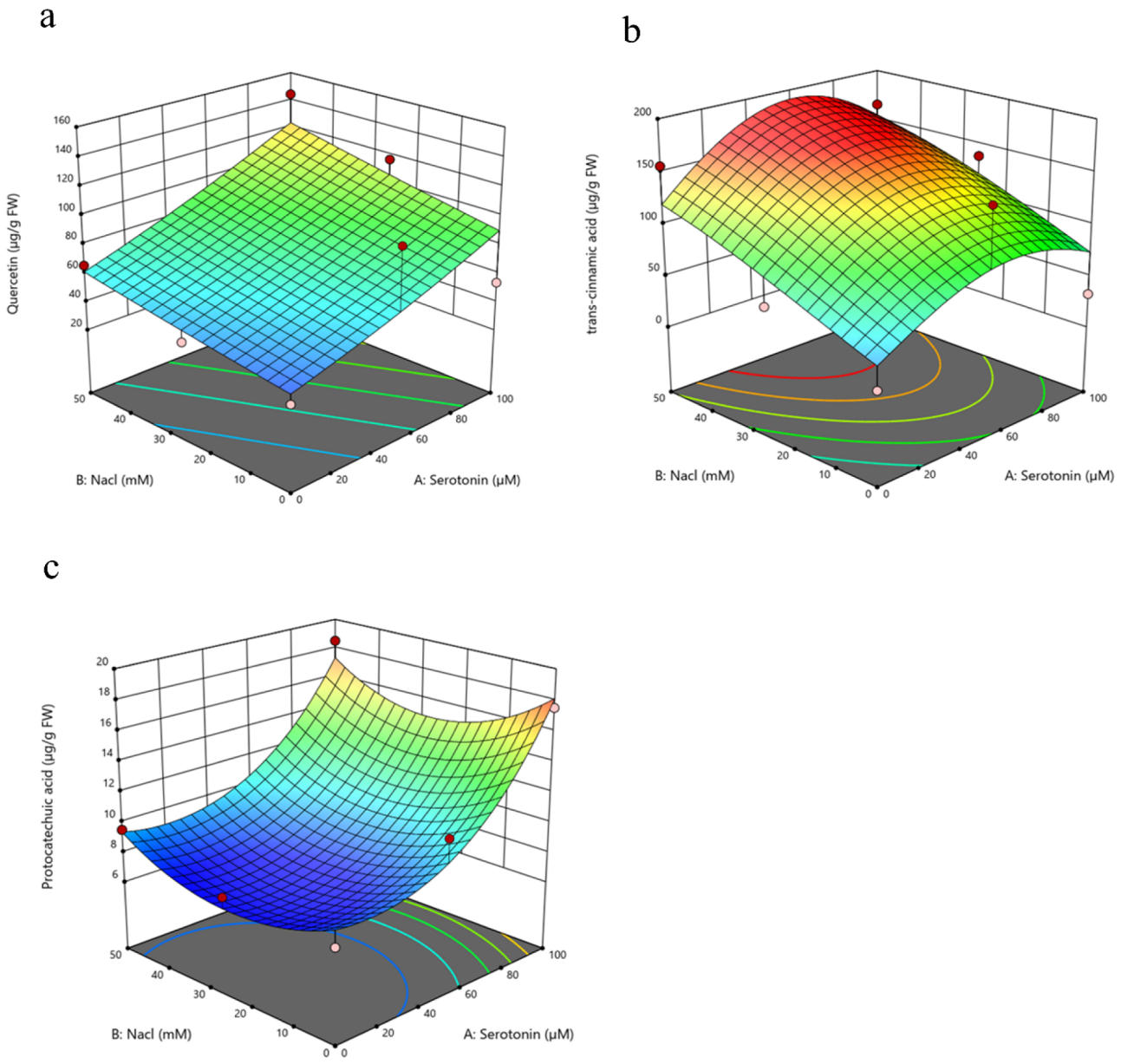

Figure 5. 3D response surface plots for the evaluation of the interactive effects of two independent variables (serotonin and NaCl) on the (a) quercetin, (b) trans-cinnamic acid, and (c) protocatechuic acid in chili cell suspension cultures.

3.6. Statistical Optimization Using RSM-CCD Model. Cell growth was acquired from nine random runs of an experimental setup to analyze the linear and quadratic model, including the pairwise and interactive effects of each variable, to study the maximum cell growth from chili cell suspension cultures. TheRSM-CCD experimental design illustrates all nine experimental runs of serotonin and NaCl variables for developing chili cell biomass (Table 2). The response values obtained from the CCD experimental design (experimental and predicted values) indicate that the serotonin-NaCl treatment is extremely affected by quadratic and linear models.

The response values were calculated from the quadratic equation for cell growth:

$$Y_1 = 1.90656 + 0.013697\ \text{serotonin} + 0.011031\ \text{NaCl} - 0.000366\ \text{serotonin} \times \text{NaCl} \quad (2)$$

In this cell growth model, Y_1 denotes the cell biomass response coefficients to the two variables, serotonin (A) and NaCl (B).

In optimization methods, the 3D response surface plots for predicting cell growth in response to serotonin and NaCl combinations are depicted in Figure 1c. The experimental data were analyzed by ANOVA, and the significance of the quadratic model and response surface was studied using the ANOVA equation obtained from the model. The ANOVA model equation indicated that the model was highly significant, fit, or did not have a probability value. At a 95% confidence level, the $p < 0.05$ represents the test's significance, and the $p < 0.001$ was the most significant. In this case, the model F value implies that the model is statistically insignificant. On the other hand, the interaction of serotonin had the highest impact on the growth of chili cell suspension cultures grown under serotonin and NaCl and was significant at $p < 0.05$, whereas the one-parameter interaction was non-significant.

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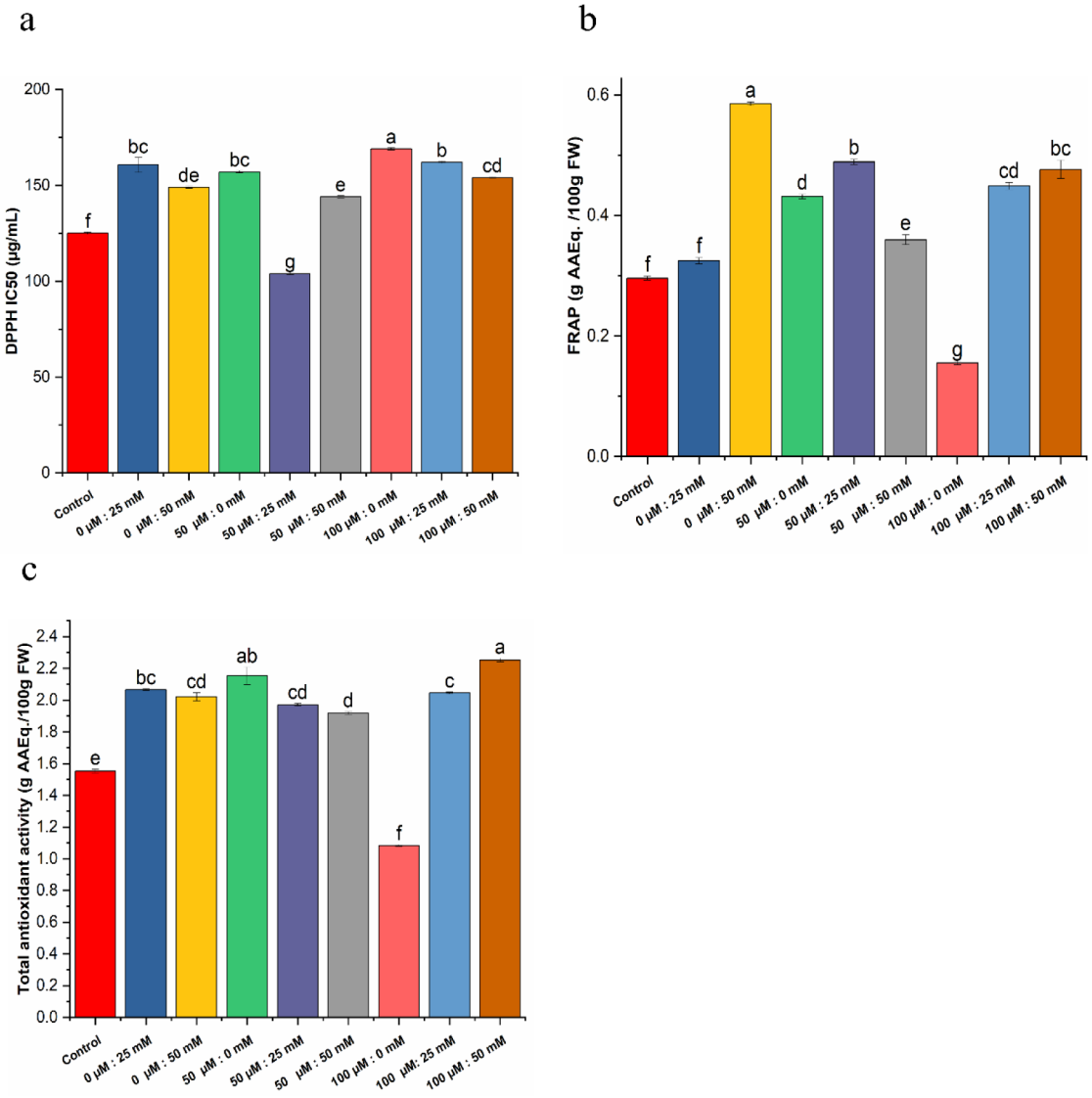

Figure 6. Effects of serotonin and NaCl on antioxidant capacity in chili cell suspension cultures: (a) DPPH, (b) FRAP, and (C) TAA. DPPH-2,2 diphenyl-1-picrylhydrazyl; FRAP-Ferric reducing antioxidant power assay; TAA-Total antioxidant activity. Data are represented as the mean ± standard deviation from three replicates. The different letters indicate significant differences from each other.

The two variables (serotonin and NaCl) effect on total phenolic content and total flavonoid content based on CCD is described in Figure 2a and b. The experimental data were evaluated using the model's coefficients and multiple regression analysis for significance levels. The linear and quadratic interaction effects of process parameters and the coefficient in terms of the two factors showed significance. The two independent variables and total phenolic content followed the quadratic equation, and the regression coefficient was ($R^{2=}$ 0.9748). The optimal levels of two independent variables on total phenolic and total flavonoid contents were estimated using three-dimensional response surface plots. The obtained quadratic equation and ANOVA are shown in Table S1. Therefore, the equation obtained to predict the total phenolic and flavonoid content using serotonin and NaCl concentration is depicted below:

$$\text{Total phenolic content} = 0.8665 + 0.0266A + 0.0168B + 0.0152AB - 0.0558A^2 - 0.0584B^2 \quad (3)$$

$$\text{Total flavonoid content} = 0.7757 + 0.0723A - 0.0655B - 0.0383AB - 0.1263\text{A}^2 - 0.0317B^2 \quad (4)$$

Serotonin and NaCl concentrations were varied according to the central composite design, and capsaicin production was measured using high-performance liquid chromatography (HPLC). The results showed that both serotonin and NaCl significantly affected capsaicin production ($p < 0.05$), and an interaction between serotonin and NaCl ($p < 0.05$) was found. Capsaicin production increased with increasing serotonin concentration, but increasing NaCl concentrations attenuated this effect. Figure 3a and b shows that both factors statistically exerted a positive response in capsaicinoids production, confirming this with the Linear model. In this regard, the

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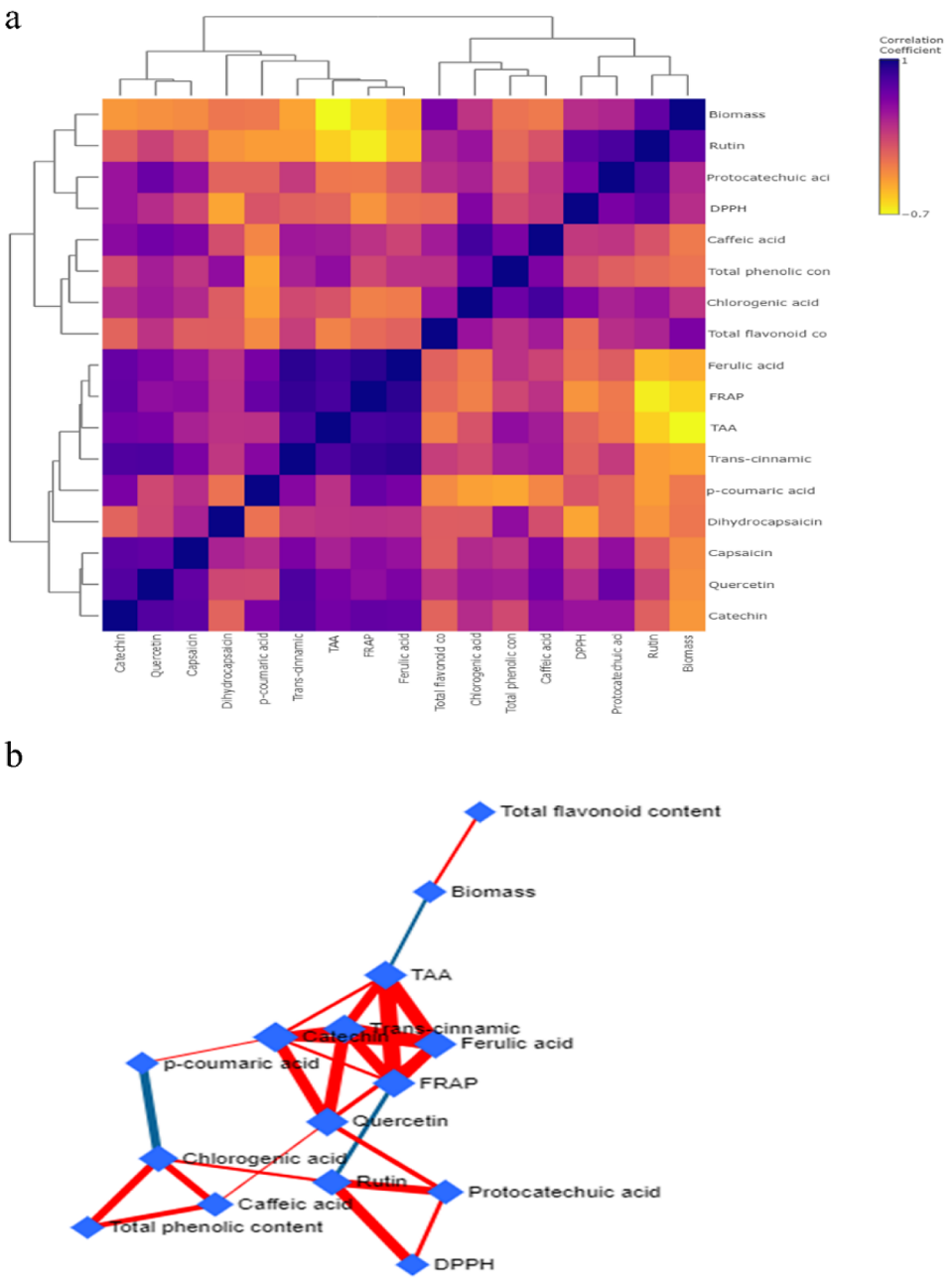

Figure 7. (a) A correlation matrix of biomass, total phenolic, total flavonoid, phenolic compounds and antioxidant capacity under serotonin and NaCl treatments. The color intensity of the matrix shows the strength of the Pearson correlation. Positive correlations are in a dark color, whil negative correlations are in a light color. (b) A correlation network was constructed between biomass, total phenolic content, total flavonoid content, phenolic compounds, and antioxidant capacity under serotonin and NaCl treatments. Nodes representing compounds measured under serotonin and NaCl treatment are shown in blue. Edges representing compounds correlated with each other, positive modes have an orange color and negative have a blue color.

effects of serotonin and NaCl parameters on the validation of the regression model were assessed using RSM-CCD. The *p*-value for all regression models indicates a probability of 0.01% when noise is present. In this model, F-values indicated the model's reliability by observing the response variance of the two factors. Herein, the independent effect of the serotonin factor was observed, substantially increasing the capsaicin content. Therefore, the obtained actual values did not match the predicted values. In addition, the interaction of two variables showed nonsignificant and lack of fit; the *F*-values and *p*-values depicted a 95% confidence level, and the probability for the regression model failed to fit the experimental data. Even though the model showed satisfactory serotonin and NaCl combination results for capsaicinoids production in suspension cultures. Similarly, the 3D response plots of the two variables show the interaction effect on the phenolic compounds, as shown in Figures 4 and 5. The linear interaction and quadratic equation of both the variables depicted for protocatechuic acid, catechin, caffeic acid, rutin, *p*-coumaric acid, ferulic acid, quercetin, and trans-cinnamic acid (Table S2). In this case, only protocatechuic acid showed a significant *p*-value, and the model *F*-value indicates that the model is significant, as shown in Table S3. Data on ferulic acid showed that the model was not significant, but $p < 0.05$, and

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the model *F*-value was too large, implying a 9.83% chance this could be due to noise. In most phenolic compound cases, it is noted that the two variables did not show significant *p* values; the interaction of the two variables was not significant, as represented in Table S3. However, there was a positive response in the production of capsaicinoids and phenolic compounds in chili suspension cultures under serotonin and NaCl application. As a result, these optimized conditions enable the industrial-scale production of pharmaceutical compounds for human health.

3.7. Correlations and Network Analyses Among Biomass and Various Metabolites. Correlation analysis evaluated the relationships among all experimental parameters (biomass, total phenolic, total flavonoids, antioxidant, capsaicinoids, and different phenolic compounds) under serotonin salt stress treatments (Figure 7a). The Pearson correlation coefficients and *p*-values showed significant positive and negative correlations between biomass and different metabolites under various serotonin and NaCl treatments (Figure 7a). The results showed a strong positive correlation between ferulic acid and trans-cinnamic acid, FRAP, and TAA ($r = 0.907^{***}$, $r = 0.902^{***}$, and $r = 0.817^{***}$). Furthermore, total phenolic content was positively correlated with chlorogenic acid and caffeic acid ($r = 0.639^{***}$, $r = 0.565^{***}$), and a negative correlation was found against p-coumaric acid, rutin and protocatechuic acid ($r = -0.372^{***}$, $r = -0.008$, and -0.067^{***}). Similarly, capsaicin showed a positive correlation against catechin, quercetin, trans-cinnamic acid, and caffeic acid ($r = 0.711^{***}$, $r = 0.685^{***}$, $r = 0.565^{***}$, and $r = 0.552^{***}$), negatively correlated with rutin and biomass ($r = 0.240^{***}$). However, FRAP showed a significant positive correlation between TAA, trans-cinnamic acid, p-coumaric acid, catechin, and capsaicin ($r = 0.794^{***}$, $r = 0.869^{***}$, $r = 0.660^{***}$, $r = 0.682^{***}$, $r = 0.504^{***}$) and negative response against rutin and biomass ($r = -0.660^{***}$, and $r = -0.557^{***}$). Moreover, a negative significant response was found between total flavonoid content against catechin, capsaicin, p-coumaric, TAA, FRAP, ferulic acid, and DPPH. Likewise, dihydrocapsaicin, rutin, and biomass showed a negative response against catechin and p-coumaric acid), and rutin showed a positive correlation between biomass, DPPH, and protocatechuic acid ($r = 0.687^{***}$, $r = 0.697^{***}$, $r = 0.789^{***}$). This study showed a maximum positive correlation with trans-cinnamic acid under different serotonin and NaCl treatments.

Figure 7b shows a correlation network plot among different metabolites and biomass in chili suspension cultures. We have chosen the *p*-values and adjusted them for correlation network analysis with a significance threshold of <0.05. The subnetwork of metabolites and biochemical targeted assays were measured, and the results were detected in positive and negative modes. Furthermore, the most matched targeted and untargeted metabolites are connected by direct edges. Moreover, in Figure 7b, the orange color shows highly correlated compounds or a positive correlation, and the blue color shows a negative correlation among the compounds. In a correlation network plot, the strength of the correlation is often indicated by the thickness or darkness of the edge. In this case, there seems to be a strong positive correlation between TAA, ferulic acid, trans-cinnamic acid, FRAP, quercetin, and catechin clustering together. Furthermore, chlorogenic acid, caffeic acid, and total phenolic content positively correlated with several other metabolites, including rutin, p-coumaric acid, catechin, and trans-cinnamic acid. Total flavonoid content showed a positive linkage with biomass and a negative correlation between biomass and TAA. Likewise, there was a strong negative correlation between p-coumaric acid and chlorogenic acid, whereas FRAP and rutin also showed a negative correlation.

4. DISCUSSION

Plant secondary metabolite production is elevated under abiotic and biotic stress to acclimatize and combat unfavorable environmental conditions. Generally, stress is associated with increased melatonin, serotonin, JA, ABA, and SA signaling molecules in plants.[12] Therefore, the present study was designed to optimize serotonin and NaCl conditions for enhancing secondary metabolite production in *in vitro* cultures by employing a response surface methodology. Interestingly, we found that RSM-optimized concentrations of serotonin and NaCl significantly influenced cell biomass, capsaicinoids, antioxidants, and phenolic compounds in suspension cultures. The results showed a significant increase in cell biomass under all the serotonin and NaCl treatments in chili cell suspension cultures compared with the control. Serotonin treatment alleviated the adverse effects of salt stress and promoted cell growth; serotonin without NaCl increased cell biomass in chili suspension cultures. Our results are consistent with the findings of Kumar et al.[12] The exogenous application of serotonin and different ratios of serotonin and melatonin stimulate cell biomass under abiotic stress by regulating gene expression associated with auxin-responsive pathways.[11,12] Conversely, cell biomass reduction was also observed in cell suspension cultures, indicating a negative effect of increased NaCl concentration with serotonin on callus biomass; similar adverse effects of salinity were also determined on seed germination in pepper seeds.[33] Similar results were found in our study, where salinity stress causes osmotic stress and ion toxicity. Changes in enzyme activity during the growth phase led to the inhibition of mineral nutrient uptake and affect the availability of ions to the cells.[5,14,33] Likewise, serotonin plays a role as a plant growth regulator; cytokinin-like activity is involved in cell division and provides an antistress effect with its antioxidative effect to promote plant growth.[33]

Compared with the control, the total phenolic and flavonoid contents were significantly enhanced by the interaction of serotonin and NaCl in chili suspension cultures. The present study observed that a higher concentration of NaCl without serotonin did not significantly affect the accumulation of the total phenolic content. In contrast, serotonin without NaCl or combined with NaCl increases the total phenolic content. Likewise, it has been reported in *Glycine max*. L, *Malus domestica*, and *Pyrus communis* that serotonin treatment increases the total phenolic content.[11,12,34] The serotonin and NaCl combination positively impacted the accumulation of total flavonoid content in chili suspension cultures. Based on these results, it can be inferred that serotonin alone or combined with NaCl at lower concentrations is responsible for a notable increase in total flavonoid content. In contrast, a higher concentration of NaCl had no significant impact on flavonoid production in suspension cultures. Similar results were reported for *Glycine max* treated with different ratios of serotonin and melatonin at various temperatures, which increased the flavonoid content.[11,12] It has been reported that serotonin interacts with the phenylpropanoid pathway and provides a substrate for the activation of phenolics and amide compounds.[35]

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Additionally, when serotonin and NaCl interact with the phenylpropanoid pathway, they activate the enzymes and elevate capsaicinoids production. Similar results were observed in our study; incorporating serotonin and NaCl altered the capsaicinoids production (capsaicin and dihydrocapsaicin) in chili cell suspension cultures. In our study, maximum capsaicin content was achieved with higher serotonin and lower NaCl treatment, and in the case of dihydrocapsaicin, lower serotonin and NaCl increased the content. Therefore, the results indicated that both cell growth and capsaicin production were triggered under higher levels of serotonin treatments. Interestingly, it is noted that a higher concentration of NaCl did not show a significant increase in capsaicinoids content and inhibited the growth as well as the capsaicinoids production in the chili cell suspension cultures. AI- Hattab et al.[5] reported similar results. Capsaicin production increased with increasing serotonin concentration, but this effect was attenuated by increasing the NaCl concentration. These results suggest that serotonin plays a crucial role in capsaicin production but NaCl can modulate its effect. Previous reports also documented that the serotonin and NaCl application in plants *ex vitro* or *in vitro*, whenever exposed to abiotic or biotic stress conditions, a cascade of signaling pathways is triggered within the cells, leading to biochemical or molecular level changes through the biosynthetic pathway genes of capsaicinoids.[36]

The most significant effects of serotonin and NaCl were observed in producing individual phenolic compounds in chili suspension cultures. Plant secondary metabolites, mainly phenolic and flavonoids, are defense molecules, potent antioxidants, stress-inducible, and help reduce ROS's detrimental effects in plant cells under serotonin and NaCl treatment.[15,37] In our findings, all phenolic compounds, such as protocatechuic acid, catechin, caffeic acid, rutin, p-coumaric acid, ferulic acid, quercetin, and trans-cinnamic acid, were affected by serotonin and NaCl treatment by stimulating the phenylpropanoid biosynthetic pathway. Moreover, it was previously reported that exogenous salicylic acid significantly increased phenolic compounds in *Capsicum annuum* seedlings[38,39] *via* upregulating the genes. In contrast, *Salvadora persica*[40] and *Lactuca sativa*[41] showed increased phenolic compounds under salinity stress. Similar to our findings, previous results illustrated that the production of phenolic acids increased under exogenous application of ABA (salicylic acid) by upregulating the expression of genes involved in metabolites biosynthesis, especially *PAL*.[38,42,43] Among quantified phenolic acids, a substantial increase of chlorogenic acid was found. These results are consistent with those of *Gardenia jasminoides* cell suspension cultures in that particularly chlorogenic acid derivatives were enhanced by cotreatment with SA and MJ.[44] Subsequently, reported in *Capsicum annuum*, phenolic acids were enhanced, such as caffeic acid, coumaroylquinic acid, ferulic acid, and chlorogenic acid, under salt stress.[45] Therefore, these findings indicate a possible enhancement of secondary metabolites production by inducing the PAL enzyme, which activates the phenylpropanoid pathway responsible for phenolic and capsaicinoid synthesis, and both are directly or closely related to each other.[45] Nonetheless, molecular analysis is necessary to better understand the modulation effects of serotonin and NaCl in chili suspension cultures. The present results suggested that serotonin and NaCl elevated the production of phenolic compounds and antioxidant capacity in suspension cultures, which is one of the beneficial effects observed. Likewise, our results also showed the potential of antioxidants under serotonin and NaCl treatment in the cultures of *Capsicum annuum*. The positive correlation between DPPH, FRAP, TAA, and phenolics in safflower under *in vitro* salinity stress confirmed this significant increase.[37] Studies indicated that serotonin, due to its phenolic group and strong antioxidant potential, boosted the efficiency of the antioxidant system, resulting in a reduction in ROS generation when plants are exposed to different biotic and abiotic stresses.[46,47] In this study, we have observed the potential and interaction of serotonin and NaCl in chili suspension cultures. It is a promising alternative approach for the *in vitro* production of secondary metabolites for therapeutic use.

5. CONCLUSION

Secondary metabolite production is significantly enhanced by reducing plant growth and development under biotic or abiotic stress. RSM-CCD optimizes serotonin's linear, quadratic interaction effect with NaCl for maximum biomass, capsaicinoids, and phenolic compounds production in chili suspension cultures. The results of the current study suggested that RSM is a reliable experimental tool for attaining optimized conditions to enhance the process conditions and reduce the time required for experiments. Results showed that both factors enhanced the cell growth, capsaicinoids, phenolic compounds, especially chlorogenic acid, and antioxidant capacity, in chili suspension cultures. The above results show a significant increase in all the desired compounds in suspension cultures. Implementing advanced techniques, such as RSM-CCD, would be a "high production-short time-low cost" formula for secondary metabolite production without affecting cell growth. In the future, the reported optimized method can be effectively used for the large-scale production of targeted metabolites in the bioreactors from a pharma-industrial viewpoint.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c05353.

The ANOVA of biomass, TPC, TFC, capsaicin, and dihydrocapsaicin content generated by CCD under the influence of serotonin and NaCl (Table S1); second-order polynomial equations generated by central composite design for the optimization of serotonin and NaCl on individual phenolic compounds and production of capsaicin and dihydrocapsaicin content (Table S2); the ANOVA of phenolic compounds production generated by CCD under the influence of serotonin and NaCl (Table S3) (PDF)

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Author Contributions

M.A., G.K., and P.G. designed and performed the experiment. M.A. and G.K. wrote the manuscript, and P.G. supervised, assisted in writing, and correction.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

2,4-D, 2,4-dichlorophenoxyacetic acid; Kin, kinetin; °C, degrees celsius; %, percentage; MS, Murashige and Skoog; h, hours; s, seconds; min, minutes; d, days; μM, micromolar; mg, milligram; g, gram; rpm, rotation per minute; R^2, coefficient of determination; JA, jasmonic acid; SA, salicylic acid; MJ, methyl jasmonate; ABA, abscisic acid; NaCl, sodium chloride; HPLC, high performance liquid chromatography; *PAL*, phenylalanine ammonia-lyase; DPPH, 2,2 diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power assay; TAA, total antioxidant activity; FW, fresh weight

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