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Vol: 1 & Iss: 1

Research Article

Conspicuous Elicitation Potential of Chitosan and Salicylic Acid on the Production of Valuable Compounds in the Callus Culture of Alcea rosea

Yawar Abbas[1]*, Masooma Jawad Hussain[1], Gohar Zaman[1], Hasnat Tariq[1], Zain Asif[1] Aneeza Rafiq[1], Marya Nawaz Malik[2], Rhuma Nisar[2], Humaira Fatima[2] and Bilal Haider Abbasi[1]

[1]Department of Biotechnology, Quaid-i-Azam University, Islamabad 45320, Pakistan

[2]Department of Pharmacy, Quaid-I-Azam University, Islamabad 45320, Pakistan

Citation: Abbas Y, Masooma Jawad H, Zaman G, et al. Conspicuous Elicitation Potential of Chitosan and Salicylic Acid on the Production of Valuable Compounds in the Callus Culture of Alcea rosea. *J Altern Complement Integra Med 2024;* 1(1): 42-50.

Received: 10 November, 2024; **Accepted:** 06 December, 2024; **Published:** 10 December, 2024

***Corresponding author:** Abbas Y, Department of Biotechnology, Quaid-i-Azam University, Islamabad 45320, Pakistan, E-mail: ayawar@bs.qau.edu.pk

ABSTRACT

All across the world, Alcea rosea is valuable for its many applications as an antioxidant, antibacterial and anticancer agent. As per established literature, for the sustainable (environmentally, economically and socially responsible) production of plant biomass and its phytochemical content, a variety of biotechnological techniques must be used. Elicitation has been demonstrated to be an extremely effective method for increasing the generation of secondary metabolites in a variety of in vitro cultures. In this study, callus cultures were exposed to various doses of chitosan and salicylic acid as elicitors. Enhancing biomass accumulation and secondary metabolite contents were the major goal. Maximum biomass accumulation for chitosan (FW: 308.25 g/L; DW: 14.75 g/L) was shown at a concentration of 10 mg/L, but for salicylic acid (FW: 281.25 g/L; DW: 14.23 g/L), the maximum biomass was seen at a substantial amount of 40 mg/L. In comparison to control and S.A-treated cultures, calli were grown on MS media supplemented with chitosan (15 mg/L) demonstrated the highest total phenolic and total flavonoid contents and production (TPC: 8.59 µg GAE/mg, TPP: 137.44 µgGAE/mg; TFC: 15.17 µg QE/mg, TFP: 242.76 µg QE/mg). With regard to antioxidant tests, cultures treated with SA (40mg/L) were detected 80.9 percent and showed the highest degree of DPPH activity as compared to CHT (15mg/L) which was detected 75.4% and control (49.4 percent). Furthermore, callus culture treated with CHT (15 mg/L) (185.3 µg AAE/mg; 168.8 µg AAE/mg) and salicylic acid (50 mg/L) (169.1 µg AAE/mg; 99.2 µg AAE/mg) revealed the highest TRP and TAC, respectively. According to the HPLC results, production was determined to be best in callus cultures made from salicylic acid as opposed to chitosan cultures and control. Salicylic acid 60 mg/L and salicylic acid 50 mg/L, which are 12 and 7-folds greater than the control (0.26 µg/mg) respectively, both increased the overall synthesis of secondary metabolites in the cultures cultivated under those conditions (total production: 3.23 µg/mg and 1.92 µg/mg, respectively). By using HPLC, catechins, which are well-known to have substantial antioxidant and anti-cancer capabilities, were also measured. Salicylic acid cultures showed optimal catechin concentrations (1.25 µg/mg), which are nearly 4 times higher than the control (0.21 µg/mg). Comparing emodin (0.51 µg/mg) to salicylic acid and control, it was the compound that was most accumulated in chitosan-derived cultures. The results showed both elicitors were very effective to increase biomass accumulation and polyphenol content in Alcea rosea callus cultures.

Keywords: Alcea Rosea, Chitosan, Salicylic Acid, Callus Culture, Phenolics, Flavonoids, TAC, TRP, Elicitation.

Abbreviations: TDZ: Thidiazuron; NAA: µ-Naphthalene acetic acid; DPPH: 2, 2-Diphenyl-1-picrylhydrazyl; DW: Dry weight; FW: Fresh weight; TFC: Total flavonoid content; TPC: Total phenolic content; TAC: Total antioxidant capacity; TRP: Total reducing potential

1. Introduction

Pharmaceutical medications are intrinsically linked to medicinal plants and the vast majority of people in the world depend on them for their health. Interest in using plant cell and tissue culture to produce commercially significant medicinal components has grown recently[1]. Among various medicinal plants, Alcea rosea (L), commonly known as garden Hollyhock, is a potential medicinal species that belongs to the Malvaceae family.

Alcea rosea (L.) is native to China, southern Europe and the Middle East. The Mediterranean and Central Asia. Expectorant, cooling, diuretic and emmenagogue were all traditional uses of A. rosea. Flowers are employed as an anti-inflammatory, febrifuge, demulcent and astringent ingredient in decoctions. Ulcers are treated with the roots of this plant. Inflammation of the kidneys and uterus can be treated with flowers as well as roots (Shah et al., 2011). Antimicrobial, cardiovascular, urolithiasis prevention, antiestrogenic, cytotoxic and immunomodulating properties were among the therapeutic properties of Alcea rosea.

A. rosea's pharmacological properties are attributed to a variety of phytochemical components. Mucilages (high molecular weight acidic polysaccharides with 1.3 to 1.6 million Dalton) were identified in Alcea rosea flowers and foliage. Glucuronic acid, galacturonic acid, rhamnose and galactose were found in these mucilages. Proteins, alkaloids, flavonoids and minerals were also present[2-4].

Its therapeutic formulations, on the other hand, are made from wild plants, so unrelated components may be present in these commercial medicines. Instead of using whole plants, recent technologies based on tissue culture technology provide options for effective phytochemical production[5]. Tissue culture techniques have been widely recognized for their ability to produce concentrated amounts of metabolites[6]. Because of totipotency, practically every cell in an in vitro culture contains the entire genetic material and is capable of producing a wider spectrum of chemicals than parental plants. As a result, growing biotechnology and genetics trends have highlighted the use of in vitro culture techniques as model systems for plant pathology, physiology, biochemistry and production of medicinally important secondary metabolites in breeding and genetics[7].

Therefore, this method can be utilized to produce phytochemicals that are either expensive to handle or difficult to obtain[8]. However, using in vitro culture technology, the primary issues connected with sustainable plant production are reduced contents, high variations and insufficient processes[9].

An increasingly common alternative for the synthesis of medicinally useful chemicals that are either produced in small quantities in wild plants or challenging to chemically synthesize in plant in vitro culture[10]. These compounds are difficult to be created chemically or are expensive. Phytochemicals, biologically active compounds found in plants, are what give them their therapeutic properties. Plants respond to various conditions by producing phytochemicals[11-13]. In response to internal and external stimuli, plants naturally create phytochemicals, although either in small amounts or only in certain parts of the plant[10]. There is still a production challenge even though plant cell and tissue culture is a good long-term manufacturing approach. For instance, elicitors can be used to help with the challenges, which is a practical way to increase the yield of these medications[14].

Elicitation, which includes different physiological and molecular components, is the process by which plants manufacture chemical defense as a stress response[15]. Both biotic and abiotic classifications apply to them[16].

In contrast to biotic elicitors, which are biological substances or agents like chitosan, chitin, polysaccharides, yeast extracts, etc.[17,18], abiotic elicitors are non-biological in nature or origin and are further classified into physical, hormonal and chemical factors (light, osmotic stress, salinity, drought stress, thermal stress, salicylic acid and gibberellic acid)[19,20].

Elicitation reduces time and yield in low-volume cultures, making it one of the most popular and economical methods for induction and boosting secondary metabolite output[21]. However, several variables, such as elicitor concentration, elicitor specificity, nutritional content, elicitor exposure time and culture age, can have an impact on elicitation[22-24].

Salicylic acid (SA), a potent plant signaling chemical, is involved in both disease resistance and growth responses[25]. by stimulating the production of defense-related molecules, SA activates a defense mechanism[26]. When apply exogenously, it imitates the interaction between the pathogen and the host and causes the expression of genes and enzymes necessary for the creation and catalysis of phenylpropanoid metabolites[27,28]. SA has been routinely used as an abiotic elicitor to enhance BAIs in plant cell culture systems[28,29].

In plant cell culture, the successful elicitation of target secondary metabolites by signal components has been observed[30]. Chitosan functions as a signal molecule in a range of plant cell cultures, triggering physiological and metabolic responses[31]. Chitosan, the second renewable source of carbon after lignocellulosic biomass, is a non-toxic biopolymer created from the alkaline deacetylation of chitin, a crucial component of fungi and arthropod cell walls[32]. Secondary metabolites like flavonoids, lignin and polyphenols are produced as a result of chitosan, which is found in a wide variety of plant species. It also induces plant defense enzymes[33,30,34]. Additionally, it was demonstrated that utilizing chitosan might be one method for reducing the negative impacts of abiotic and biotic stresses[35].

Hence, the present study aimed to inspect the potential effects of SA and CHT as an elicitor for the increase of biomass yield and enhancement of secondary metabolites including total phenolic, flavonoids and polyphenols in the callus culture of Alcea rosea.

2. Materials and Methods

2.1. Germination of Seeds and Callogenesis

Alcea rosea seeds were acquired from the Quaid-e-Azam University, Islamabad's Plant Cell Culture Laboratory (PCCL) seed bank. The seeds were first treated with alcohol and $HgCl_2$, then three times with autoclaved dH_2O[36]. The pH of the culture medium was set to 5.65 to 5.68 prior to autoclaving. After seeds were inoculated into flasks, they were incubated at a temperature of 25 ± 2 °C for 8/16 h (dark/light) with a light intensity of 40 mol m^{-2} s^{-1}. As tissue culture had previously been optimized by[37], leaf explants (derived from in vitro plantlets) were cultivated on MS media fortified with 1 NAA and 0.1 TDZ for the callus

culture (Hasnat et al., 2022). After inoculation media flask with leaf explants, the flasks were then shifted to the culture room having optimum conditions for callus growth.

2.2. Elicitor Preparation

In order to prepare the elicitors, the method of khan et al., 2019 was used. CHT (chitosan) was dissolved in 0.1M acetic acid and then stirred for 5 hours at 60 °C temperature. 1mM Salicylic acid was dissolved in distilled water and stirred for 5 hours for proper mixing of salicylic acid[16]. Various concentrations of chitosan (0, 1, 5, 10, 15, 20 and 30 mg/L) and salicylic acid (0, 10, 20, 30, 40, 50 and 60 mg/L) were used in MS (Murashige and Skoog) media along with 1 mg/L NAA and 0.1 mg/LTDZ and 30 g/L sucrose for elicitation experiments. Each flask was inoculated with approx. 0.5 grams of callus. A triplet was used for each concentration and then placed for 28 days in the growth room.

2.3. Sample Extraction

Calli were extracted from the cultured media on Whitman filter paper after 28 days and dried at room temperature for 20 minutes to eliminate any extra moisture adhered to the callus. In order to serve drying purposes, the study established the weight of fresh calli while keeping it at 48°C for 24 hours. Using a motor and pestle, dried calli were crushed into a fine powder.

To prepare the extract, 0.1 g of callus dry powder and 0.5 ml of methanol were mixed and vortexed for 5 minutes, followed by 30 minutes of sonication with a sonicator (USC1200TH) and finally 15 minutes of centrifugation at 12,000 rpm[39].

2.4. Total Phenolic Content (TPC)

The assay was carried out in a 96-well plate. In each well of a 96-well plate, a 20 µL of sample from each extract's 4 mg/ml stock solution was added. A 90 µL of FC reagent was then added. After 30 minutes of incubation at 37°C, 90 µL of sodium bicarbonate was added to each well on the plate. The optical density of the sample extracts was measured at 630 nm using a microplate reader. To create a calibration curve, gallic acid was used as a positive control in two-fold serial dilutions (2.5, 5, 10, 20, 40 µg/ml) and methanol as a negative control. The experiment's findings were represented as µg of gallic acid equivalent (GAE) per milligram of the extract.

2.5. Total Flavonoid Content (TFC)

The 96-well plate was used to conduct the assay. An aliquot of 20 µL from the test extracts (4 mg/ml) was added to each well, then 160 µL of distilled water, 10 µL of potassium acetate and 10 µL of aluminum chloride. The absorbance of the test extracts at a wavelength of 415 nm was assessed using a microplate reader after 30 minutes of incubation at room temperature. The calibration curve for this test was developed using quercetin at different doses (2.5, 5, 10, 20 and 40 µg/ml) as the positive control and methanol as the negative control. The results were calculated using the formula µg of extract/g quercetin equivalent (QE)/mg.

2.6. Total Reducing Power (TRP)

The reducing power of the extracts was computed using a potassium ferricyanide colorimetric test. A 250 µL potassium ferricyanide (1 percent w/v in distilled water) was combined with 200 µL of phosphate buffer (0.2 M, pH 6.6) and an aliquot of 100 mL of each test extract (4 mg/ml methanol) before being incubated at 50 °C for 20 minutes in a water bath. Each test chemical was mixed with 200 µL of trichloroacetic acid (10 percent w/v in distilled water) and the mixture was centrifuged at 3000 rpm for 10 minutes at room temperature. The 50 µL of 0.1 percent w/v FeCl3 in distilled water on a 96-well plate were then added to the 150 µL of supernatant. The absorbance was then determined at 630 nm. Methanol acted as the negative control and ascorbic acid (1 mg/ml) served as the positive control. Ascorbic acid equivalent (AAE) was used to measure each sample's reducing power, which is given as µg AAE/mg (i.e. µg ascorbic acid equivalent per mg).

2.7. Total Antioxidant Capacity (TAC)

Using a phosphomolybdenum-based method, the test sample's overall antioxidant capacity was ascertained. The TAC reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate) was mixed with 900 µL of test extract (4 mg/ml methanol) and 100 µL of test extract. Methanol served as the negative control. The reaction mixture was then heated to 95 degrees Celsius and placed in a water bath for 90 minutes. After reaching 95 degrees Celsius, the reaction mixture was submerged in water for 90 minutes. The absorbance of the test and standard solutions was assessed at 630 nm after cooling. The antioxidant activity was calculated as the ascorbic acid µg equivalents per milligram of dry weight (µg AAE/mg of DW).

2.8. DPPH Free Radical Scavenging Assay

The compounds' antioxidant capacity was tested using the stable free radical 2, 2-diphenyl-1-picrylhydrazyl (DPPH). A 10 µL of plant extract (4 mg/ml methanol) and 190 µL of DPPH solution (9.6 mg/100 methanol) were combined in order to achieve the reaction mixture's final concentrations of 200 µg/ml. The absorbance at 517 nm was measured using a microplate reader after 30 minutes of incubation at 37°C. To calculate the percentage of free radical scavenging activity, the study uses the formula below.

$$\text{Free Radical Scavenging Activity} = 1 - As/Ac * 100$$

Where, As and Ac, respectively, stand for the absorbance of the sample and the adverse control. Ascorbic acid was utilized as a positive control in the test, which was carried out in three iterations.

2.9. High-Performance Liquid Chromatography Analysis

Shimadzu HPLC system with controller (SCL-10AVP), degasser (DGU-14A), low-pressure mixer (FCV-10ALVP), pump (LC-10AT VP) in combination with 3D-PDA detector (SPD-M10A VP) and a software (LC solution) was employed to perform the RP-HPLC based analysis of polyphenols (total 13). Agilent Zorbax C8 (5 m; 4.6 250 mm) column was utilized. The binary gradient system was used to detect the polyphenols, employing mobile phase A (methanol: water: acetic acid: acetonitrile) in a ratio of (10:85:1:5) and mobile phase B (acetonitrile: methanol: acetic acid) in a ratio (40: 60: 1). At 1.2 ml/min, the flow rate was changed. 50 l was the injection volume. Furthermore, the gradient volume of the mobile phase B was 0-75 percent in the first 0–30 min, 75–100 percent in the next 0–31 min, 100 percent in the next 0–35 min and 0–36 min. The column underwent a 10-minute reconditioning process before the new sample was injected. Methanol was present in each standard at a concentration of 50 g/ml and the sample was made by dissolving 100 l in 1 ml of methanol. Polyphenol concentrations were calculated by comparing the UV absorption spectra and the retention duration of the sample to the reference.

2.10. Statistical Analysis

Each of the aforementioned trials was carried out in duplicate, synchronized and twice. To formalize statistical analysis, the mean value was determined in each experiment and the standard error was calculated (Microsoft Excel Program). All figures were created using the Origin program (8.5).

3. Results and Discussions

3.1. Effects of Chitosan and Salicylic Acid on Biomass Accumulation

Throughout the course of a plant's life cycle, salicylic acid is engaged in the regulation of vital biochemical and physiological parameters (Rivas-San Vicente and Plasencia, 2011). However, CHT has an impact on plant signal transduction pathways like Nitric oxide (NO) signals and phenylalanine ammonia lyase and it may assist regulate gene expression due to its intimate connection with chromatin. CHT and SA, as well as plant endogenous enzymes like SOD that aid in the deactivation of ROS's negative effects, are necessary for the increased production of phenolic and flavonoid chemicals[39].

Biomass accumulation (fresh and dry weights) of A. rosea was examined after applying different concentrations of CHT (1, 5, 10, 15, 20, 30 mg/L) and SA (10, 20, 30, 40, 50, 60 mg/L) along with control having no elicitors. On the 28th day of inoculation, callus culture was harvested. In comparison to control, there was an increase in biomass production. In the case of Salicylic acid, the maximum biomass accumulation (fresh weight 281.25 g/L and dry weight 14.75 g/L) was seen with the 40 mg/L scales. At 60 mg/L of SA, the lowest result (157.25 g/L) was noted. The higher SA concentrations were revealed to prevent callus proliferation. This might be as a result of stress put on by increased SA concentrations[40].

Increased SA concentrations dramatically stress growth of cell, according to Bulgakov et al. (2002), causing less biomass to accumulate in Rauwolfia cordifolia callus culture. SA had only minimal influence on cell proliferation and biomass accumulation in callus cultures of grape and Rosa hybrid[41-43].

Salvia miltiorrhiza cell cultures' proliferation was observed to be modestly reduced by SA treatment in previous work (Li et al., 2003). The growth of Rubia cordifolia callus cultures was likewise found to be significantly constrained by raising the SA levels in the media[44]. The current research is in agreement with previous reports[45].

In the case of Chitosan, the maximum biomass accumulation (308.25 g/L) was observed at the CHT (10 mg/L) and the lowest (165.91 g/L) value was recorded at CHT (30 mg/L). **(Figure 1, Figure 2 and Figure 3)** Based on the amount, CHT may have a negative impact on the growth of biomass, although this effect is counter balanced by the nutritional advantages of CHT[46,47].

The application of chitosan causes increased biomass accumulation, as it has the capacity to increase hydration and nutrition uptake by managing the osmotic pressure inside the cell[48,49]. Different in vitro culture approaches for diverse plant species, including callus culture of flax[50] and Fagonia indica, as well as cell suspensions of different species of Basil[48] and red sage (Salvia miltiorrhiza)[51], have all been proven to increase the formation of biomass.

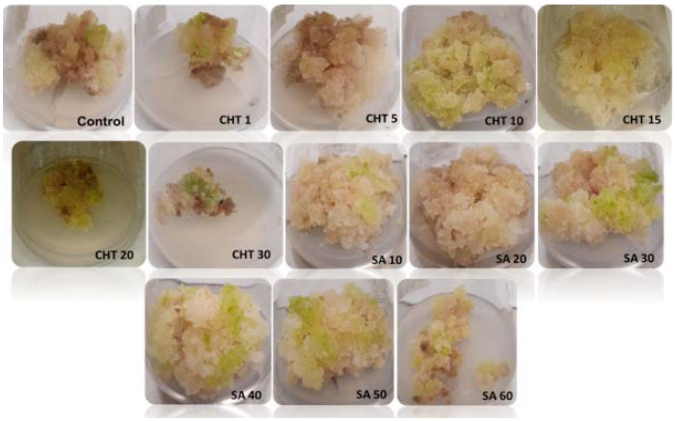

Figure 1: Morphology of A. rosea callus cultures grown in different concentration of CHT and SA.

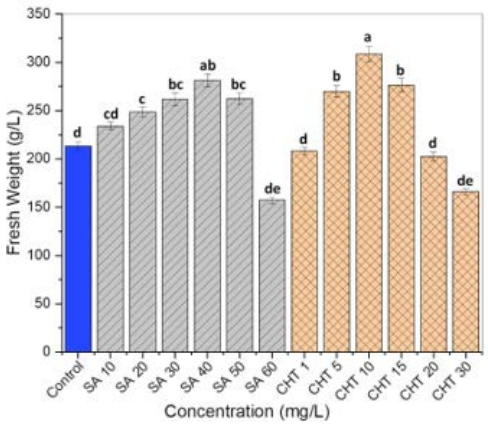

Figure 2: Fresh weight of A. rosea callus cultures, grown in different concentration of CHT and SA. Values are the mean ± SD of 3 independent replicates. The different letters deficit the statistical differences among various experimental conditions ($p<0.05$).

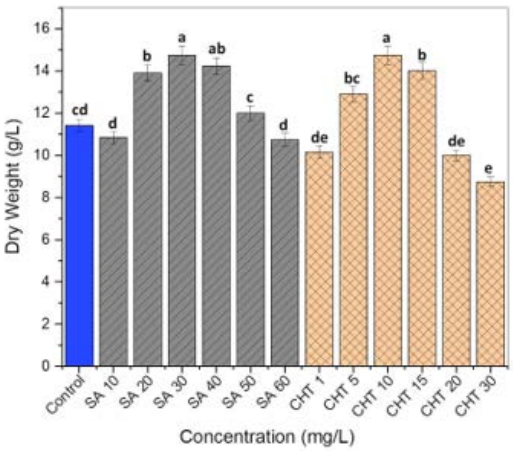

Figure 3: Dry weight of A. rosea callus cultures, grown in different concentration of CHT and SA. Values are the mean ± SD of 3 independent replicates. The different letters deficit the statistical differences among various experimental conditions ($p<0.05$).

3.2. Effects of Chitosan and Salicylic Acid on Total Phenolic and Flavonoids Contents

Low molecular weight compounds like flavonoids and phenols serve vital roles in plant physiology and growth. They are the most discussed classes of chemicals due to their immense

potential for acting as signaling molecules, offering defense against pathogenic attacks and controlling essential biochemical processes, such as antioxidant activity[52].

In the present study, phenolics and flavonoid contents were investigated in chitosan and salicylic acid derived cultures. Callus grown on MS media supplemented with chitosan (15 mg/L) showed maximum TPC, TPP, TFC and TFP (TPC: 8.59 µg GAE/mg, TPP: 137.44 µgGAE/mg; TFC: 15.17 µgQE/mg TFP: 242.76 µgQE/mg) as compared to control (TPC: 3.05 µgGAE/mg, TFC: 7.48 µgQE/mg, TPP: 39.78 µgGAE/mg, TFP: 97.45 µgQE/mg) as shown in **(Figure 4 and Figure 5)**.

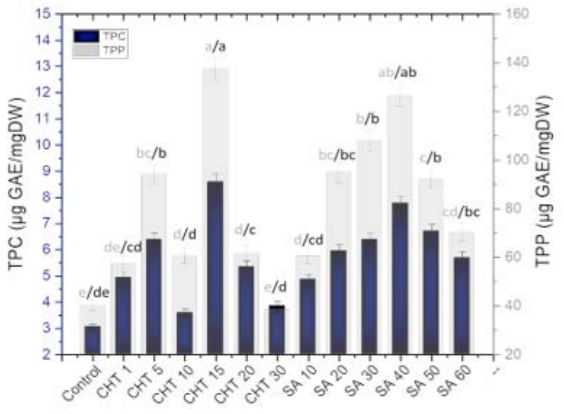

Figure 4: TPC and TPP of A. rosea callus cultures grown in different concentration of CHT and SA. Error bar represents the means deviation of triplicates. Values are the mean ± SD of 3 independent replicates. The different letters deficit the statistical differences among various experimental conditions ($p<0.05$).

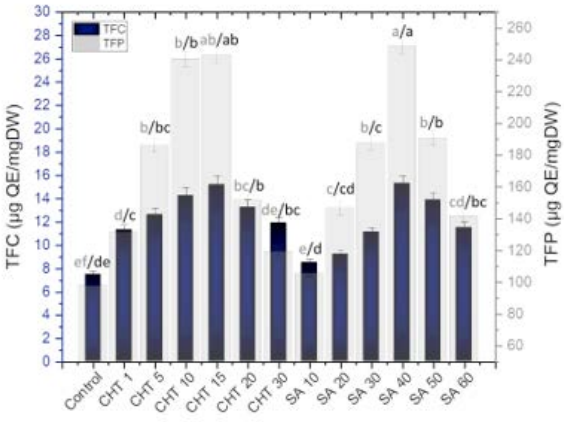

Figure 5: TFC and TFP of A. rosea callus cultures grown in various concentration of CHT and SA. Error bar represents the means deviation of triplicates. Values are the mean ± SD of 3 independent replicates. The different letters deficit the statistical differences among various experimental conditions ($p<0.05$).

The increased production of phenolic and flavonoids may be due to CHT's ability to mimic naturally occurring fungal pathogenicity and therefore, induce plant defense mechanisms. Chitosan causes a number of defense responses, such as an increase in cytosolic H^+ and Ca^{2+}, activation of MAP-kinases, callose apposition, oxidative burst, hypersensitivity response (HR) and the synthesis of phytoalexins, jasmonate, abscisic acid (ABA) and proteins associated with disease (PR). CHT's effects on plant signal transduction pathways like phenylalanine ammonia-lyase and nitric oxide signals may also help control gene expression[53].

The synthesis of phenolic and flavonoids under different salicylic acid applications on callus cultures of A. rosea was also studied. When compared to the control, salicylic acid (40 mg/L) significantly increased the accumulation of TPC (7.77 mg/g), TPP (126.36 mg/L) and (TFC: 15.30 gGAE/mg TFP: 248.75 gGAE/mg). The stimulation of PAL activity and metabolic pathways are potential explanations for the enhanced TPC buildup. A series of substantial branch phenols are created as the yield increases[54]. For instance, it was discovered that production was biomass-dependent and a positive association between phenolic and flavonoid production as well as biomass accumulation was noticed.

Similar findings have been made by other studies that have found that SA has a positive effect on flavonoid production in a number of plant species, which is consistent with the study findings[55,56]. In these investigations, elevated expression of the genes and enzymes required for the synthesis of flavonoids was linked to the accumulation of flavonoids, which may also be the scenario in the present instance.

The augmented production of phenolic and flavonoid molecules, as well as plant endogenous enzymes like SOD that aid in the deactivation of ROS's damaging effects, are all facilitated by CHT and SA. Additionally, SA treatment enhances PAL activity, which results in increased phenolic compound accumulations[57].

3.3. Antioxidant Assays (DPPH, TAC, TRP)

When plants are under stress, they produce a significant amount of ROS, which damages essential organic molecules and restricts the growth and development of plants[58]. However, plants contain a wonderful defense mechanism that utilizes low molecular weight antioxidant substrates and an antioxidant enzyme system to mitigate the detrimental effects caused by ROS[59].

One of the most popular types of these chemicals that act as free radical scavengers are phenolic compounds. Antioxidant assays including DPPH, TAC and TRP were used to evaluate antioxidant activity. Due to its high sensitivity, moderate clarity and practicability, the DPPH test is a widely used technique for assessing the antioxidant potential of the products derived from plant cells. The existence of both ET- and/or HAT-based pathways for antioxidant activity is thought to be detectable by DPPH (2,2-diphényl-1-picrylhydrazyle), which is then used to calculate the %age of free radicals that the sample is able to scavenge[60].

In SA, the optimal DPPH activity was found (40mg/L) (80.9%) and CHT (15mg/L) (75.4%) in treated cultures as compared to control (49.4%) (Fig. 4.6). This is due to the fact that flavonoids and phenolics account for the majority of plants' antioxidant potential[61,62]. According to a report, in order to defend cells from ROS, SA activated several antioxidant enzyme genes, including those for SODs, catalases and ascorbate peroxidases. Moreover, highest TRP and TAC was detected in callus culture treated with CHT (15mg/L) i.e. (185.3 µg AAE/mg; 168.8 µg AAE/mg) and salicylic acid (50mg/L) i.e. (169.1 µg AAE/mg; ٩٩,٢ µg AAE/mg), respectively and this could be aligned with higher secondary metabolite production **(Figure 6, Figure 7 and Figure 8)**.

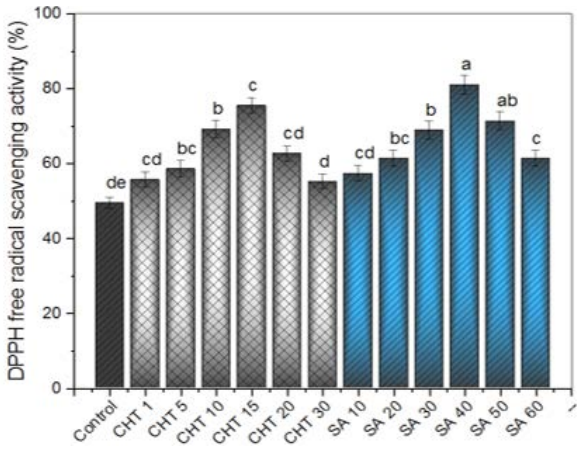

Figure 6: DPPH-FRSA activity of A. rosea callus cultures grown in various concentrations of CHT and SA. Error bar represents the means deviation of triplicates. Values are the mean ± SD of 3 independent replicates. The different letters deficit the statistical differences among various experimental conditions ($p<0.05$).

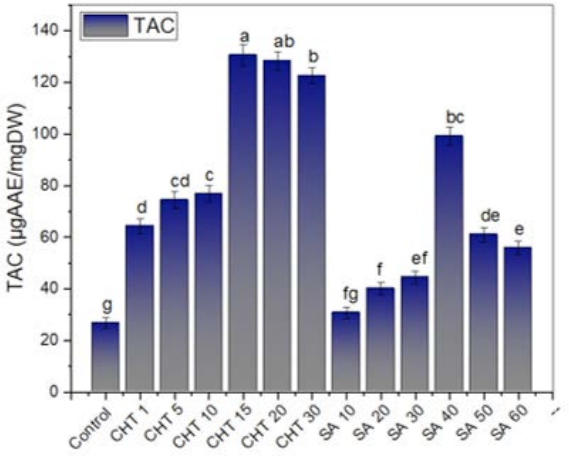

Figure 7: Total antioxidant capacity (TAC) of A. rosea callus cultures grown in various concentrations of CHT and SA. Error bar represents the means deviation of triplicates. Values are the mean ± SD of 3 independent replicates. The different letters deficit the statistical differences among various experimental conditions ($p<0.05$).

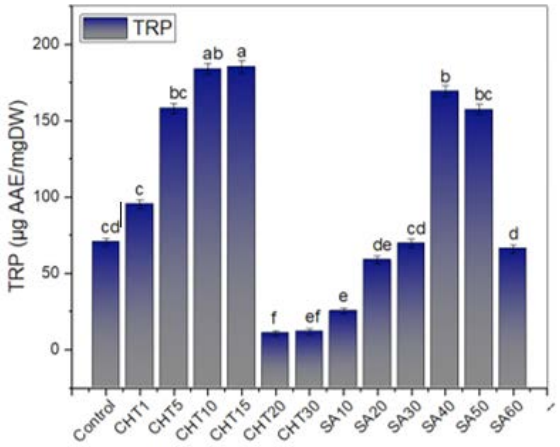

Figure 8: Total reducing power (TRP) of A. rosea callus cultures grown in various concentrations of CHT and SA. Error bar represents the means deviation of triplicates. Values are the mean ± SD of 3 independent replicates. The different letters deficit the statistical differences among various experimental conditions ($p<0.05$).

3.4. Quantification of Polyphenols in Alcea rosea Callus Culture by RP-HPLC

A sophisticated method for evaluating secondary metabolites is HPLC. A. rosea callus cultures produced from chitosan and salicylic acid were measured in the current investigation for a total of 13 polyphenolics. The HPLC results demonstrate that the salicylic acid-derived callus cultures were found to yield the highest level of polyphenolics as compared to chitosan-derived cultures and control. The maximum amount of these chemicals could be produced in cultures that were grown in salicylic acid 60 mg/L (total production: 3.23 µg/mg) followed by salicylic acid 50 mg/L (total production: 1.92 µg/mg) which are 12 and 7 folds higher than control (0.26 µg/mg), respectively.

The production levels in salicylic acid 40 mg/L (1.67 µg/mg), chitosan 1 mg/L and chitosan 10 mg/L (1.72 µg/mg and 1.13 µg/mg, respectively) cultures were also found feasible while chitosan 30 mg/L and 20 mg/L cultures had the least impact on the generation of secondary metabolites **(Table 1)**.

Table 1: RP-HPLC-based quantification of polyphenols in callus culture of Alcea rosea.

		Polyphenols quantified (µg/mg Dry weight)											
Treatment	Conc.	**Vanillic acid**	**Rutin**	**Catechin**	**Syringic acid**	**Coumeric acid**	**Emodine**	**Gentisic acid**	**Caffeic acid**	**Ferulic acid**	**Cinnemic acid**	**Luteolin**	**Apigenin**
Control		0.016 ± 0.0017	0.14 ± 0.01	0.212 ± 0.02	0.031 ± 0.0018	0.003 ± 0.0008	0.429 ±0.018	0.013 ±0.0012	0.027 ± 0.0016	0.004 ± 0.0006	0.006 ± 0.0014	0.082 ± 0.012	0.02 ± 0.0017
	1	0.004 ±0.001	0.029 ± 0.003	0.251 ± 0.03	0.007 ± 0.0012	0.013 ± 0.0013	0.486 ± 0.019	0.005 ± 0.0009	0.012 ± 0.0013	0.004 ± 0.0006	0.028 ± 0.0018	0.532 ± 0.018	0.035 ± 0.0018
	5	0.0003 ± 0.0007	0.088 ±0.007	0.055 ± 0.016	0.004 ± 0.0011	0.009 ± 0.0011	0.317 ± 0.015	0.022 ± 0.0015	Nd	0.0009 ± 0.0004	0.0006 ± 0.001	0.008 ± 0.001	0.0009 ± 0.001
	10	0.029 ± 0.0018	0.097 ± 0.008	0.267 ± 0.03	0.025 ± 0.0016	0.037 ± 0.002	0.294 ± 0.014	0.042 ± 0.002	0.047 ± 0.002	0.028 ± 0.001	Nd	0.141 ± 0.013	0.053 ± 0.0019
	15	Nd	0.07 ± 0.006	0.193 ± 0.02	0.003 ± 0.001	0.007 ± 0.001	0.518 ± 0.02	0.035 ± 0.0018	0.004 ± 0.0006	0.003 ± 0.0005	0.008 ± 0.0014	0.216 ± 0.015	0.018 ± 0.0016
	20	0.001 ± 0.0009	0.039 ± 0.004	0.089 ± 0.018	0.016 ± 0.0014	0.03 ± 0.0018	0.115 ± 0.011	0.031 ± 0.0017	0.009 ± 0.0011	0.004 ± 0.0006	Nd	0.012 ± 0.012	9E-04 ± 0.001
Chitosan	30	0.001 ± 0.0009	0.056 ± 0.005	0.042 ± 0.015	0.004 ± 0.001	0.025 ± 0.0017	0.118 ± 0.011	0.011 ± 0.0011	0.005 ± 0.0007	0.002 ± 0.0004	0.002 ± 0.0012	0.024 ± 0.013	0.002 ± 0.0013
	10	0.004 ± 0.001	0.018 ± 0.002	0.087 ± 0.018	0.035 ± 0.002	0.016 ± 0.0015	0.146 ± 0.013	0.02 ± 0.0014	0.045 ± 0.0019	0.005 ± 0.0007	0.008 ± 0.0014	0.171 ± 0.014	0.019 ± 0.0016
	20	0.0003 ±0.0007	0.02 ± 0.002	0.184 ± 0.02	0.009 ± 0.0013	Nd	0.142 ± 0.012	0.018 ± 0.0013	0.03 ± 0.0017	0.003 ± 0.0005	0.008 ± 0.0014	0.08 ± 0.012	0.005 ± 0.0014

	30	0.0004 ±0.0007	0.034 ± 0.004	0.028 ± 0.014	0.007 ± 0.0012	0.002 ± 0.0007	0.156 ± 0.013	0.005 ± 0.0009	0.013 ± 0.0013	0.00008 ±0.0003	0.005 ± 0.0013	0.044 ± 0.011	0.004 ± 0.0014
	40	0.0004 ±0.0007	0.062 ± 0.005	0.713 ± 0.06	0.005 ± 0.0012	0.021 ± 0.0016	0.485 ± 0.019	0.026 ± 0.0016	0.006 ± 0.0008	0.0003 ±0.0002	0.011 ± 0.0015	0.15 ± 0.013	0.028 ± 0.0017
	50	0.005 ± 0.001	0.094 ± 0.007	0.574 ± 0.05	0.031 ± 0.0018	0.027 ± 0.0017	0.409 ± 0.017	0.003 ± 0.0007	0.007 ± 0.0009	0.006 ± 0.0008	0.013 ± 0.0016	0.32 ± 0.016	0.018 ± 0.0016
Salicylic acid	60	0.036 ± 0.002	0.12 ± 0.009	1.254 ± 0.08	0.002 ± 0.0009	0.011 ± 0.0012	0.368 ± 0.016	0.008 ± 0.001	0.008 ± 0.001	0.01 ± 0.0009	0.043 ± 0.002	0.75 ± 0.02	0.072 ± 0.002

In the present research work, the quantification of compounds through HPLC demonstrates that the optimal catechins concentrations (1.25 µg/mg) were found in salicylic acid-derived cultures as compared to the chitosan and is about 4 folds greater than control (0.21 µg/mg). Catechins have sufficient antioxidant and cancer-preventing properties. The most prevalent molecules in the callus cultures of Uncaria elliptica and Fagopyrum esculentum were epicatechin and epicatechin-3-O-gallate, respectively, according to recent investigations on the generation of catechins in vitro[63,64]. However, on the contrary, enhanced catechins production was reported in chitosan-treated cultures than salicylic acid in Fagonia indica.

As for chitosan-derived cultures, the highest accumulated compound was emodin (0.51 µg/mg) as compared with salicylic acid and control. Emodin contains various medicinal properties including, anticancer, hepatoprotective, anti-inflammatory, antioxidant and antibacterial actions, according to newly available research[65].

These substances, for example, have significant pharmacological significance. According to earlier research, these metabolites have an anticancer effect by inhibiting intercellular communication, suppressing oncogenes, decreasing antioxidative forces, inducing apoptosis in a variety of cell lines and reducing antioxidative forces[66]. Additionally, interactions between these polyphenolics and enzyme systems such as protein kinases and polymerases as well as cytochrome p540 have been discovered to have substantial anticancer properties[67].

4. Conclusions

As a consequence of the application of elicitors, the A. rosea callus cultures produced enhanced biomass and bioactive compounds. The callus cultures derived from CHT had increased concentrations of phenolics, flavonoids and antioxidant activity. Additionally, it was shown that the SA acid promoted the production of phytochemicals and biomass growth. SA-induced cultures produced metabolites more efficiently than CHT and control. According to the current findings, elicitors, particularly CHT, have a great potential for increasing polyphenolic chemicals in callus culture and producing biomass. From the aforementioned findings, it is implied that A. rosea callus cultures developed in different chitosan and concentrations of salicylic acid offer a consistent source for increased production of plant secondary metabolites with significant medicinal and industrial uses. The current research presents a competent protocol for high-frequency production of secondary metabolites that paves the way for elicitation of these metabolites in the cell suspension culture of A. rosea in the future. Without destroying the natural plant habitat, this method can be used while generating a larger mass of bioactive compounds in a short amount of time to address the rising demand for metabolites.

5. Author Contributions

Y.A performed experiments, TPC, TFC, DPPH, TAC and TRP activities. M.J.H assisted Y.A with major experiments and other biochemical assays. H.T, A.R and Z.A assisted Y.A in sample preparations. G.Z assisted Y.A with TPC, TFC and DPPH. M.N.M, R.N and H.F performed extraction for HPLC and conducted HPLC. B.H.A conceived the idea, supervised the research and reviewed the paper critically.

6. Compliance with Ethical Standards

Conflict of Interest

The authors declare that they have no conflict of interest.

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