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Evaluation of the Effect of Chitosan/Cinnamon Essential Oil Hydrogel Formulation on Biochemical Traits (Total Phenol, Flavonoid, and Antioxidant Enzyme Activity) of Pepper Plant Against the Fungus *Alternaria Solani*

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ABSTRACT: This study evaluated the inhibitory effects of cinnamon essential oil, chitosan, and their combination against *Alternaria solani*, as well as their impact on disease severity, phenolic and flavonoid content, and the activity of defense-related enzymes peroxidase (POD) and superoxide dismutase (SOD). Based on the analysis of inhibitory percentages, the positive control, Cin.Ch. 500 ppm, and chitosan 600 ppm were identified as the most effective treatments for suppressing fungal growth, with mean inhibition rates of 93.66%, 93.33%, and 95.00%, respectively. Analysis of variance (ANOVA) revealed that treatment type, concentration, and their interaction significantly influenced disease severity ($p < 0.05$). The combined treatment of 600 ppm cinnamon and chitosan (Cin.Chi 600 ppm) showed the lowest mean disease severity (19.67), representing the most effective strategy for disease reduction. Regarding flavonoid and phenol content, treatment had a significant effect on flavonoids ($F=550.501$, $p<0.001$) and on phenols ($F=3.538$, $p=0.038$). Day significantly affected both flavonoids ($F=23.530$, $p<0.001$) and phenols ($F = 46.506$, $p<0.001$) and concentration parameter significantly affected flavonoids ($F = 260.453$, $p < 0.001$) and phenols ($F=4.438$, $p=0.041$). However, the interaction between treatment and concentration significantly affected flavonoids ($F=19.621$, $p<0.001$) but did not significantly affect phenols ($F=2.638$, $p=0.106$). Regarding defense enzymes, all treatments of cinnamon essential oil, chitosan, and their combination significantly affected POD and SOD activity, and the interaction between day and treatment also had a notable impact. Overall, the Cin.Chi 600 ppm treatment exhibited the greatest effect on both POD and SOD activity across the three examined days, indicating that increasing the concentration of both components to 600 ppm enhances the stimulation of defense enzyme activity. In conclusion, the application of Cin.Chi 600 ppm, incorporating both cinnamon and chitosan, serves as an effective strategy for enhancing POD and SOD defense enzyme activity, increasing flavonoid and phenol content, and reducing disease severity caused by *Alternaria solani*.

1. INTRODUCTION

Pepper (*Capsicum annuum* L.), a member of the Solanaceae (nightshade) family, is an economically important horticultural crop widely cultivated in tropical and subtropical regions [1, 2]. Originating from Central and South America, *C. annuum* is characterized by green stems, dark-green ovate leaves, and a plant height ranging from approximately 30 to 150 cm [3]. The fruits exhibit considerable diversity in size, shape, color, and pungency, occurring in red, green, yellow, and other color variations. Morphological forms range from spherical and bell-shaped to elongated and highly pungent types, reflecting the extensive genetic diversity within the species. Owing to its distinctive flavor and nutritional value, pepper is widely utilized as both a vegetable and a culinary spice [4]. In addition to its agricultural and culinary significance, *C. annuum* is recognized as a rich source of bioactive phytochemicals and antioxidant defense components [1]. The fruit contains a wide range of biochemical compounds, including carotenoids, phenolic compounds, flavonoids, anthocyanins, and saponins, which contribute to its nutritional and functional properties [5]. These phytochemicals possess strong antioxidant activity and play a crucial role in scavenging reactive oxygen species (ROS) and other free radicals, thereby reducing oxidative damage to cellular components [5]. Furthermore, pepper tissues contain several antioxidant enzymes, including catalase (CAT), peroxidase (POD), superoxide dismutase (SOD), and glutathione peroxidase (GPX) [6]. These enzymes constitute an important enzymatic defense system that mitigates oxidative stress by catalyzing the conversion of reactive oxygen intermediates, such as superoxide radicals and hydrogen peroxide, into less harmful molecules, including water and molecular oxygen. Numerous studies have reported that regular consumption of pepper and pepper-derived phytochemicals may contribute to the prevention of chronic diseases associated with oxidative stress, including cardiovascular disorders, certain types of cancer, and metabolic diseases [7]. Therefore, *C. annuum* can be regarded as a valuable dietary source of bioactive compounds and antioxidant enzymes with significant potential for promoting human health and reducing the adverse effects of free radical-induced cellular damage. Pepper leaf spot, caused by the fungus *Alternaria solani*, is recognized as one of the most significant diseases affecting pepper (*Capsicum* spp.) worldwide [8]. The disease causes substantial damage in many pepper-producing countries, including Iran. Although pepper leaf spot is widespread across the country, it triggers serious yield losses in specific regions, depending on factors such as climatic conditions, fungal strain, disease management practices, and pepper cultivar. Comprehensive national data on the exact percentage of yield loss attributed to this disease in Iran remain unavailable; moreover, the extent of damage is likely to vary with local environmental conditions and cultivation practices. On a global scale, pepper leaf spot also imposes considerable production losses due to its widespread occurrence. These losses manifest as reduced yield and diminished fruit quality. To manage and control the disease, an integrated approach combining physical, biological, and chemical methods is typically employed. Cinnamon essential oil is a volatile plant-derived oil extracted primarily from the bark, leaves, or other tissues of cinnamon species (*Cinnamomum* spp.). It is widely recognized for its characteristic aroma, distinctive flavor, and diverse biological activities. Due to its natural origin and broad spectrum of functional properties, cinnamon essential oil has attracted considerable attention in the food, pharmaceutical, cosmetic, and nutraceutical industries [9]. The chemical composition of cinnamon essential oil varies depending on the plant species, geographical origin, extraction method, and plant part utilized [9]. However, cinnamaldehyde is generally considered the predominant constituent, accounting for the characteristic aroma and flavor of cinnamon and contributing significantly to its biological activity. Other important compounds include eugenol, a phenolic constituent known for its antimicrobial and antifungal properties, and various terpenes such as limonene and β -caryophyllene, which enhance the oil's aromatic profile and may contribute to its therapeutic potential [9]. Numerous studies have demonstrated that cinnamon essential oil possesses a wide range of biological activities, including antimicrobial, antifungal, antioxidant, anti-inflammatory, and insecticidal properties [10]. Chitosan is a naturally occurring polysaccharide derived primarily from the deacetylation of chitin, a structural biopolymer found in the exoskeletons of crustaceans, insects, and certain fungal cell walls. Chemically, chitosan consists of β -(1 $\rightarrow$ 4)-linked D-glucosamine and N-acetyl-D-glucosamine units. Due to its biodegradability, biocompatibility, non-toxicity, and diverse biological activities, chitosan has attracted considerable attention in agriculture, biotechnology, medicine, and food science [11]. One of the most notable characteristics of chitosan is its broad-spectrum antimicrobial activity against various microorganisms, including bacteria, fungi, and certain viruses [12]. In addition to its antimicrobial effects, chitosan functions as a natural elicitor capable of inducing plant defense responses and enhancing tolerance to both biotic and abiotic stresses [13, 14]. Numerous studies have demonstrated that chitosan can stimulate antioxidant defense mechanisms in plants by increasing the activity of key antioxidant enzymes involved in the detoxification of ROS. Among them, SOD plays a crucial role in the first stage of antioxidant defense by catalyzing the conversion of superoxide radicals into hydrogen peroxide and molecular oxygen. Enhanced SOD activity contributes to the reduction of oxidative damage caused by excessive reactive oxygen species accumulation [15]. Chitosan

treatment has also been reported to increase POD activity, an enzyme involved in hydrogen peroxide detoxification, cell wall strengthening, and plant defense responses against environmental stressors and pathogen attack [16]. CAT is another important antioxidant enzyme whose activity may be enhanced following chitosan application. CAT rapidly decomposes hydrogen peroxide into water and oxygen, thereby preventing the accumulation of toxic levels of reactive oxygen species within plant cells [17]. As a result, plants treated with chitosan often exhibit enhanced tolerance to a wide range of environmental stresses, including drought, salinity, temperature extremes, heavy metal toxicity, and pathogen infection [18]. By strengthening antioxidant defense systems and reducing oxidative damage, chitosan contributes to improved plant growth, physiological performance, and stress resilience. Therefore, chitosan can be regarded as an effective natural biostimulant and elicitor that enhances plant defense mechanisms through the activation of antioxidant enzymes and the maintenance of oxidative–antioxidative balance. The aim of the proposed research is to evaluate the effect of chitosan/cinnamon essential oil hydrogel formulation on biochemical parameter (total phenol, flavonoid, and antioxidant enzyme activity) of pepper plant against the fungus *Alternaria solani*

2. EXPERIMENTAL

2.1. Fungus preparation

Alternaria solani was obtained from the Mycology Collection of the Science and Research Branch, Islamic Azad University, Tehran.

2.2. Host plant

Pepper seeds were sown in plastic pots filled with steam-sterilized soil (65°C) and maintained under controlled greenhouse conditions at 24°C until the desired growth stage. After three weeks, plants at the four-leaf stage were selected for the experiments.

2.3. Preparation of fungal inoculum

A single-spore isolate of *Alternaria solani* recovered from pepper, whose pathogenicity had been confirmed in preliminary assays, was cultured on potato dextrose agar (PDA) medium. The culture was maintained at $20 \pm 4^\circ\text{C}$ with an 8-hour light / 16-hour dark photoperiod. Following two weeks of growth, conidia were dislodged from the agar surface using 10 mL of sterile distilled water and a coverslip. The resulting inoculum was counted with a hemocytometer and subsequently diluted to the required concentration.

2.4. Preparation of chitosan gel

To prepare the chitosan gel, 1 g of chitosan was added to 100 mL of 1% (v/v) acetic acid. After 30 minutes of stirring, a uniform and homogeneous chitosan solution was obtained, which constituted the chitosan gel.

2.5. Preparation of cinnamon plant essential oils

Essential oil was extracted from cinnamon plants via steam distillation using a Clevenger apparatus. Preliminary tests determined that the stem bark contained the highest essential oil content among plant organs; therefore, stem bark was used for extraction. Approximately 100 g of powdered stem bark was placed in the distillation flask with 500 mL of distilled water and heated for 4 hours. Following distillation, the essential oil, having a lower density than water, floated to the surface and was collected by draining the aqueous phase from the bottom valve of the apparatus. To obtain larger volumes of essential oil, the procedure was repeated multiple times. The collected oil was stored in amber glass bottles at 4°C to prevent photo-oxidation.

2.6. Biochemical evaluations

2.6.1. Evaluation of defensive enzyme activities

For enzyme extraction and assay, frozen leaf samples were placed in a porcelain mortar, covered with liquid nitrogen, and ground thoroughly until completely pulverized. One gram of the resulting powder was transferred into 2 mL microcentrifuge tubes, and 2 mL of phosphate buffer (pH 6.7) was added. The mixture was vortexed and immediately centrifuged at 14,000 rpm for 15 minutes at 4°C. Following centrifugation, the supernatant (enzyme extract) was collected and kept on ice throughout the subsequent analysis. This extract was used for assaying PPO activity.

2.6.1.1. Assay of PPO enzyme activity

SOD activity was assayed according to the method of Giannopolitis and Ries (1977) by measuring the inhibition of nitroblue tetrazolium (NBT) photoreduction at 560 nm [19]. A 50 mM potassium phosphate buffer (pH 7.5) was prepared. The reaction mixture consisted of 50 mM phosphate buffer (pH 7.5), 0.1 mM EDTA, 75 μ M NBT, 13 mM methionine, and 4 μ M riboflavin, prepared freshly and kept in the dark. For each sample, 100 μ L of enzyme extract was added to 3 mL of the reaction mixture in a test tube. The reaction was initiated by illuminating the tubes with a 40 W fluorescent lamp. After 8 minutes of illumination, absorbance was measured at 560 nm. A light control consisting of 3 mL of reaction mixture without enzyme extract was similarly illuminated. In the absence of SOD, NBT photoreduction proceeded to 100%, converting all NBT to formazan; the absorbance of this control represented complete photoreduction. One unit of SOD activity was defined as the amount of enzyme required to inhibit 50% of NBT photoreduction. The percent inhibition for each sample was calculated based on the difference in absorbance between the sample and the light control. SOD activity was then expressed as units per gram of fresh weight.

2.6.1.2. POD enzyme activity

Peroxidase activity was assayed using a reaction mixture consisting of 2 mL of phosphate buffer (0.2 M, pH 4.8), 200 μ L of 3% (v/v) H_2O_2 , and 200 μ L of 0.04 M benzidine dissolved in 50% (v/v) methanol. The enzyme extract was then added to the reaction mixture, and the increase in absorbance at 530 nm per minute was measured using a spectrophotometer. Enzyme activity was expressed as the change in absorbance at 530 nm per minute per gram of fresh weight, according to the method of Chance and Maehly (1995) [20].

2.6.1.3. Measurement of total flavonoids

Total flavonoid content was determined using the aluminum chloride colorimetric method. The reaction mixture was prepared by sequentially adding 0.1 mL of 10% (w/v) aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water to test tubes. Subsequently, 0.5 mL of the methanolic plant extract was added. After thorough mixing, the absorbance of the reaction mixture was measured at 415 nm using a colorimeter. Total flavonoid content was quantified using a calibration curve constructed with quercetin as the standard and expressed as quercetin equivalents per gram of fresh weight (or appropriate unit).

2.7. In vitro evaluations

2.7.1. Effect of the optimal essential oil concentration for inhibiting fungal growth

The antifungal activity of the prepared cinnamon essential oil was evaluated in vitro at six concentration levels: 100, 200, 300, 400, 500, and 600 mg/L. Two control treatments were included: a positive control consisting of PDA medium supplemented with the fungicide carbendazim, and a negative control consisting of unsupplemented PDA medium. The dependent variable was the percentage of mycelial growth inhibition at each concentration. Additionally, the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of the cinnamon essential oil were determined.

2.7.2. Effect of the optimal chitosan gel concentration for inhibiting fungal growth

The antifungal activity of chitosan gel against *Alternaria solani* was evaluated using the poisoned food (medium mixing) method. Erlenmeyer flasks containing PDA were autoclaved and then cooled to 42–45°C. Chitosan gel was added to the molten PDA at concentrations of 100, 200, 300, 400, 500, and 600 mg/L, and the mixture was stirred thoroughly to form a uniform emulsion. The amended media were immediately poured into 8 cm diameter Petri dishes and allowed to solidify. A 5 mm diameter mycelial disc was excised from the margin of a, actively growing culture of *A. solani* using a sterile cork borer and placed centrally in each Petri dish.

The inoculated plates were incubated at 25°C. After 24 hours, colony diameters were measured daily until the negative control plates (containing unamended PDA without chitosan) were completely overgrown by the fungus. Each treatment was replicated three times, with three Petri dishes per replicate. A control treatment (PDA without chitosan gel) was also included. The percentage of mycelial growth inhibition for each chitosan concentration was calculated using Abbott's formula (Abbott, 1925) as represented in Equation 1.

Equation 1

$$\text{Nanoparticle inhibition percentage} = \frac{\text{Control colony diameter} - \text{Treatment colony diameter}}{\text{Control colony diameter}} \times 100$$

2.8. In vivo evaluations

2.8.1. Pathogenicity studies in the pathosystem (plant and pathogen)

2.8.1.1. Preparation of inoculum (fungal inoculum preparation)

A 10-day-old culture of *Alternaria solani* grown on PDA was used to prepare the spore suspension. Sterile distilled water containing Tween 80 (1% v/v) was added to the culture, and the plate was agitated vigorously to release conidia into suspension. The spore concentration was determined using a hemocytometer and adjusted to 2×10^5 conidia/mL. This suspension was subsequently used for pathogenicity assays.

2.8.2. Effect of formulated hydrogel/essential oil on controlling *Alternaria* leaf spot

To evaluate the effect of the formulated hydrogel containing cinnamon essential oil on increasing tomato resistance to *Alternaria* leaf spot disease, tomato plants were grown in the laboratory until the five-leaf stage. Effective concentrations of the hydrogel formulation for controlling the pathogenic fungus were selected based on the in vitro antifungal assays described above. Three plants were assigned to each concentration.

Plant treatments with the formulated hydrogel/essential oil were applied in two regimes: (1) prior to fungal inoculation (preventative treatment) and (2) following fungal inoculation (curative treatment). The effect of each treatment regime on disease resistance was evaluated. For each treatment, disease severity and disease incidence were calculated and recorded.

2.9. Evaluation of biochemical changes and disease resistance level in the host plant

For disease resistance evaluation, plants were inoculated with the pathogen 24 hours after treatment with the nanoparticles formulated in chitosan hydrogel. Disease severity and disease incidence were assessed seven days post-inoculation to determine the level of induced resistance resulting from the nanoparticle treatment. To evaluate biochemical changes in the plants, tissue sampling was conducted at 1, 3, and 6 days after nanoparticle treatment, and enzymatic activities were analyzed.

2.10. Statistical population, sampling method, and sample size

The experiment was arranged as a completely randomized design (CRD). Each treatment consisted of three replicates, with one replicate defined as one pot containing three seedlings. Two control treatments were included: a positive control (pepper seedlings inoculated with the pathogenic fungus) and a negative control (pepper seedlings treated with sterile distilled water without any test compound). Data were analyzed using one-way analysis of variance (ANOVA), and mean comparisons were performed using Duncan's multiple range test (DMRT) at a significance level of $p < 0.05$ using SPSS software.

3. RESULTS AND DISCUSSION

3.1. Inhibitory effect of cinnamon essential oil and chitosan on the growth of *A. solani* fungus under in vitro conditions

In this research, cinnamon essential oil and chitosan were considered as natural compounds, and their effects on the growth of *A. solani* were evaluated. For this purpose, laboratory experiments were conducted under in vitro conditions. The results of statistical analysis showed that both cinnamon essential oil and chitosan possess significant inhibitory potential against the growth of *A. solani* under in vitro conditions ($p < 0.001$) (Table 1). These results suggest that the use of cinnamon essential oil and chitosan can serve as an effective inhibitory method for controlling the growth of *A. solani* in plants.

To obtain the optimal results, the mean growth inhibition percentages for each treatment were examined as represented in Fig. 1. Based on the results, the positive control and Cin.Ch.500 ppm treatments yielded the highest efficacy, with a mean inhibition of 93.66%. To compare all treatments, we consider their mean inhibition percentages, leading to the following conclusion: The mean inhibition value of chitosan (62.63%) is higher than that of cinnamon essential oil (55.36%).

This result indicates that chitosan generally has a greater effect on reducing the growth of the fungus *Alternaria solani* compared to cinnamon essential oil. When comparing the different cinnamon essential oil treatments, it can be observed that the inhibition value of the treatment Cin.Ch.500 ppm (93.33%) is higher than that of cinnamon essential oil at other concentrations. Also,

among the different chitosan treatments, chitosan at 600 ppm showed the highest inhibition percentage (95.00%). The positive control treatment also yielded favorable results, with a mean inhibition of 93.66%. Accordingly, the positive control and Cin.Ch.500 ppm treatments exhibited the best inhibition percentages. Based on the comparison of mean inhibition values: for cinnamon essential oil: the best treatment was Cin.Ch. 600 ppm (91% inhibition), followed by Cin.Ch. 500 ppm (93% inhibition). For chitosan: the best treatment was 600 ppm (95% inhibition), followed by 500 ppm (86% inhibition).

Table 1. Analysis of variance of the effect of cinnamon essential oil, chitosan, and their combination on the growth inhibition percentage

ANOVA					
Inhibition	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	44397.333	15	2959.822	356.963	.000
Within Groups	265.333	32	8.292		
Total	44662.667	47			

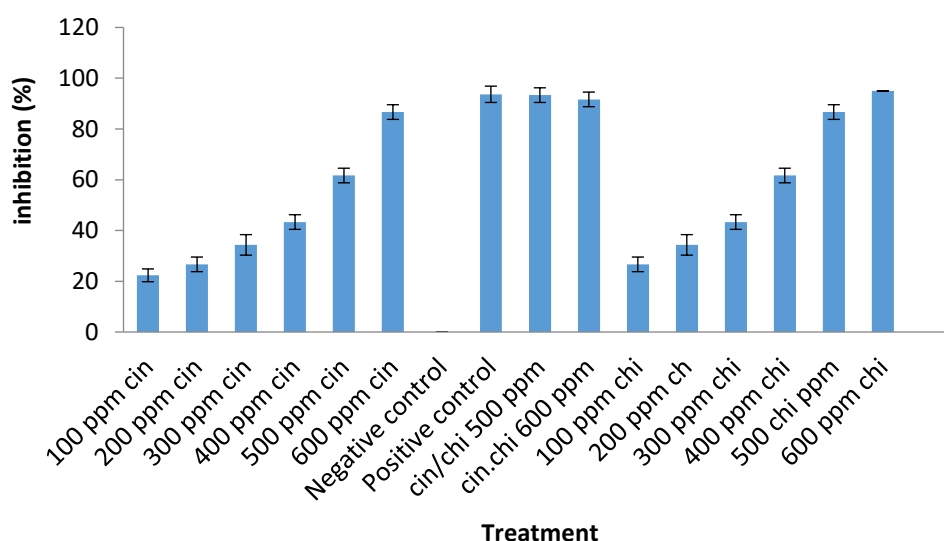


Fig. 1. Effect of different levels of cinnamon essential oil, chitosan and their mixture treatments on the inhibition percentage

3.2. Inhibitory potential of cinnamon essential oil and chitosan against disease progression caused by *A. solani* under in vivo conditions

The results of the ANOVA test for the disease severity indicate that there is a significant effect of the treatment type and concentration, as well as their interaction, on disease severity ($P < 0.05$, Table 2). For the variable treatment type, the p-value is 0.000, indicating that treatment type has a significant effect on disease severity.

Regarding the variable concentration, the p-value is also less than 0.05 ($p\text{-value} < 0.05$), indicating that concentration parameter also has a significant effect on disease severity. Furthermore, the interaction between treatment type and concentration has a significant effect on disease severity, as the p-value for is 0.014 ($p\text{-value} < 0.05$). From this analysis, it can be concluded that treatment type, concentration, and the interaction between these two variables have a considerable effect on disease severity.

Table 2. Analysis of variance table for the effect of cinnamon essential oil, chitosan, and their combination (cinnamon/chitosan) on the control of *Alternaria* leaf spot disease caused by *A. solani*

Tests of Between-Subjects Effects

Dependent Variable: Disease.severity

Source	Type III Sum of Squares	Df	Mean Square	F	Sig.
Treatment	81.000	1	81.000	19.440	.000
Concentration	50920.889	5	10184.178	2444.203	.000
Treatment * Concentration	75.333	5	15.067	3.616	.014
Error	100.000	24	4.167		

a. R Squared = .998 (Adjusted R Squared = .997)

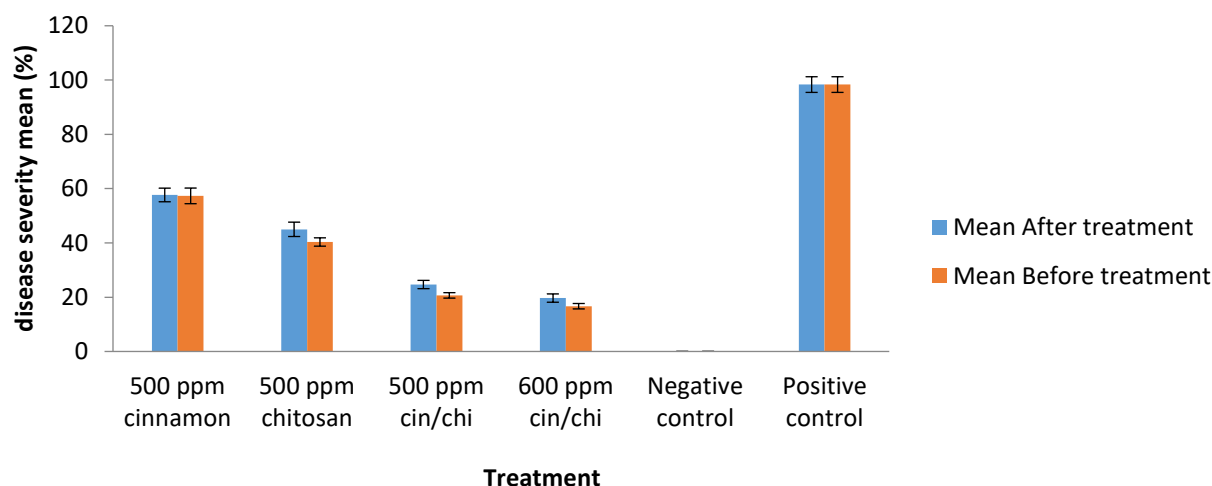


Fig. 2. Duncan's mean comparison chart for the effect of different treatments on disease severity of leaf spot caused by the fungus *A. solani*

Pairwise comparisons revealed several statistically significant differences among treatments. Specifically, the treatment combining 500 ppm cinnamon with 500 ppm chitosan showed a significant difference ($p < 0.05$). The combination of 500 ppm cinnamon with the 600 ppm cinnamon/chitosan treatment also yielded a significant difference ($p < 0.05$). Similarly, the treatment of 500 ppm chitosan when compared with 600 ppm cinnamon/chitosan was significantly different ($p < 0.05$). Relative to the negative control, all tested treatments demonstrated significant differences: 500 ppm cinnamon ($p < 0.05$), 500 ppm chitosan ($p < 0.05$), 500 ppm cinnamon/chitosan ($p < 0.05$), and 600 ppm cinnamon/chitosan ($p < 0.05$). These results indicate that all applied treatments had a measurable effect relative to the negative control, and that several pairwise comparisons among active treatments also reached statistical significance. According to the obtained results, the lowest disease severity is related to the treatment 600 ppm cin/chi, with a mean of 19.66. This result indicates that the use of the combination of 600 ppm cinnamon and chitosan has a significant effect on reducing disease severity. Therefore, the treatment 600 ppm cin/chi is introduced as the best treatment for reducing disease severity.

3.3. Effect of cinnamon essential oil, chitosan, and chitosan/cinnamon treatments on phenolic and flavonoid content of the plant

According to the ANOVA test (Table 3), treatment had a statistically significant effect on both flavonoid and phenolic content. For the flavonoid variable, the analysis yielded an F-value of 550.501 and a p-value of less than 0.001 (Sig. = .000), indicating a

highly significant effect of treatment. For the phenolic variable, the F-value was 3.538 with a p-value of 0.038, which also indicates a significant effect of treatment, albeit with a lower level of statistical significance compared to flavonoids. Regarding the effect of day, the ANOVA test (Table 3) revealed significant effects on both flavonoid and phenolic content. For the flavonoid variable, the F-value was 23.530 with a p-value of less than 0.001, indicating that day significantly influences flavonoid content. Similarly, for the phenolic variable, the F-value was 46.506 with a p-value of less than 0.001, demonstrating that day also exerts a significant effect on phenolic content. The effect of concentration was also assessed via ANOVA (Table 3). For the flavonoid variable, the F-value was 260.453 with a p-value of less than 0.001, indicating that concentration has a significant effect on flavonoid content. For the phenolic variable, the F-value was 4.438 with a p-value of 0.041, demonstrating that concentration also significantly affects phenolic content, though to a lesser degree than flavonoids. Regarding the interaction between day and concentration (the day $\times$ concentration), the ANOVA test (Table 3) revealed no significant effect for either variable. For both flavonoid and phenolic content, the F-value was 0.489 with a p-value of 0.617, indicating that the day $\times$ concentration interaction does not significantly influence flavonoid or phenolic content. Similarly, the interaction between treatment and day (treatment $\times$ day) was also found to have no significant effect on either flavonoid or phenolic content. For both variables, the ANOVA test (Table 3) yielded an F-value of 0.540 with a p-value of 0.707, indicating that the treatment $\times$ day interaction does not significantly influence flavonoid or phenolic content. In contrast to the non-significant interactions reported above, the interaction between treatment and concentration (treatment $\times$ concentration) was found to have a significant effect on both flavonoid and phenolic content. For the flavonoid variable, the ANOVA test (Table 3) yielded an F-value of 134.159 with a p-value of less than 0.001, indicating a highly significant treatment $\times$ concentration interaction. For the phenolic variable, the F-value was 3.583 with a p-value of 0.037, demonstrating that the interaction between treatment and concentration also significantly affects phenolic content, albeit with a lower level of significance compared to flavonoids. Based on the above results, it can be concluded that treatment, day, and concentration each have significant effects on flavonoid and phenolic content. Furthermore, the interaction between treatment and concentration also significantly affects both flavonoid and phenolic content. In contrast, the interactions between day and concentration, as well as between treatment and day, do not have significant effects.

Table 3. ANOVA table for the effect of treatment, day, and concentration on flavonoid and phenolic content in different samples

Source	Dependent Variable	Type III Sum of Squares	Df	Mean Square	F	Sig.
Treatment	Flavonoid	12.520	2	6.260	550.501	.000
	Phenol	82.384	2	41.192	3.538	.038
Day	Flavonoid	.535	2	.268	23.530	.000
	Phenol	1082.960	2	541.480	46.506	.000
Concentration	Flavonoid	2.962	1	2.962	260.453	.000
	Phenol	51.672	1	51.672	4.438	.041
Day * Concentration	Flavonoid	.011	2	.006	.489	.617
	Phenol	387.069	2	193.535	16.622	.000
Treatment * Day	Flavonoid	.025	4	.006	.540	.707
	Phenol	223.119	4	55.780	4.791	.003
Treatment * Concentration	Flavonoid	3.051	2	1.526	134.159	.000
	Phenol	83.445	2	41.723	3.583	.037
Error	Flavonoid	.455	40	.011		
	Phenol	465.732	40	11.643		

3.4. Results of Duncan's multiple range test for the effect of different treatments on phenol and flavonoid values

Fig. 3 examines the effect of two treatment factors—chitosan and chitosan/cinnamon—at two different concentrations (500 ppm and 600 ppm) on flavonoid and phenolic content. For flavonoids, at a concentration of 500 ppm, the chitosan/cinnamon treatment produced the highest mean flavonoid content (1.284), followed by chitosan alone (0.967) and cinnamon alone (0.665). At a concentration of 600 ppm, chitosan/cinnamon again yielded the highest mean value (2.419), compared to chitosan (1.179) and cinnamon (0.724), representing a substantial increase. For phenolics, at a concentration of 500 ppm, the chitosan/cinnamon treatment resulted in a mean phenolic content of 19.029, which was higher than chitosan alone (15.359) and cinnamon alone (12.753). At a concentration of 600 ppm, chitosan/cinnamon also produced the highest mean value (18.882), compared to chitosan (15.241) and cinnamon (14.288). Therefore, it can be concluded that the use of chitosan/cinnamon at both 500 ppm and 600 ppm leads to an increase in mean flavonoid and phenolic content. Furthermore, the use of chitosan alone also exerts a positive effect on the mean values of these two variables.

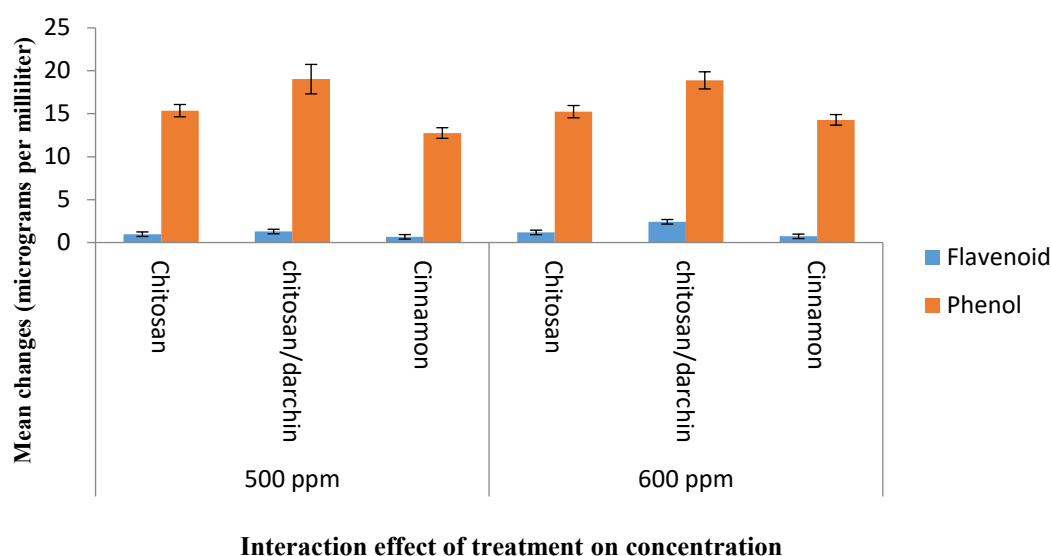


Fig. 3. Duncan's mean comparison test results of the interaction effect of treatment and concentration on changes in phenol and flavonoid concentrations

According to the data presented in Fig. 4, the following conclusions can be drawn regarding the effects of chitosan/cinnamon compared to chitosan and cinnamon alone across three days. For flavonoid: On day 1, the use of chitosan/cinnamon (mean: 1.706) showed a significant difference compared to chitosan (mean: 0.972) and cinnamon (mean: 0.580) ($p < 0.05$). On day 2, chitosan/cinnamon (mean: 1.832) also demonstrated a significant difference relative to chitosan (mean: 1.075) and cinnamon (mean: 0.704) ($p < 0.05$). On day 3, similarly, chitosan/cinnamon (mean: 2.016) differed significantly from chitosan (mean: 1.801) and cinnamon (mean: 0.704) ($p < 0.05$). For phenol: on day 1, the use of chitosan/cinnamon (mean: 7.980) showed a significant difference compared to chitosan (mean: 11.799) and cinnamon (mean: 12.915) ($p < 0.05$). On day 2, chitosan/cinnamon (mean: 16.333) showed no significant difference compared to chitosan (mean: 17.514) and cinnamon (mean: 14.841) ($p > 0.05$). On day 3, chitosan/cinnamon (mean: 23.553) again showed a significant difference compared to chitosan (mean: 20.586) and cinnamon (mean: 15.306) ($p < 0.05$). According to the statistical analysis performed, the mean flavonoid content on days 1, 2, and 3 all showed a significant increase with the use of chitosan/cinnamon. For phenolic content, significant increases were observed on days 1 and 3, whereas on day 2, the difference in mean phenolic content with the use of chitosan/cinnamon was not significant.

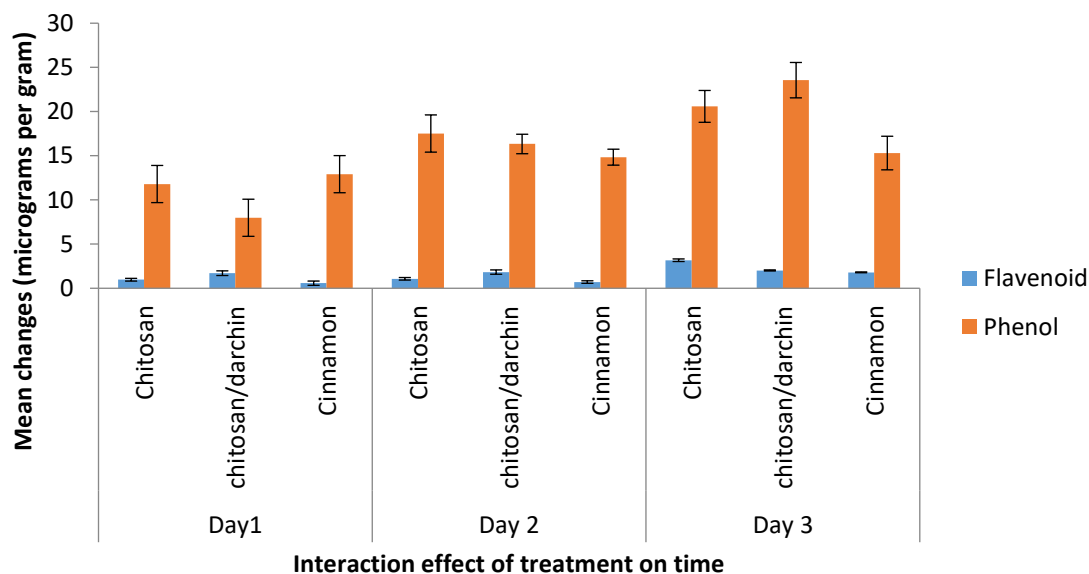


Fig. 4. Results of Duncan's multiple range test for the interaction effect of treatment and time on changes in phenol and flavonoid concentrations

By comparing all treatments with one another (Fig. 5), the following conclusions are drawn. For the flavonoid variable, the chitosan/cinnamon treatment (mean: 1.852) was significantly higher than the chitosan treatment (mean: 1.073), while the cinnamon treatment (mean: 0.695) was significantly lower than both of the other treatments.

For the phenolic variable, the chitosan/cinnamon treatment (mean: 18.956) was significantly higher than the chitosan treatment (mean: 15.300), and the cinnamon treatment (mean: 13.021) was significantly lower than both of the other treatments. Therefore, it can be concluded that the use of the chitosan/cinnamon treatment, compared to the chitosan and cinnamon treatments alone, leads to a significant increase in mean flavonoid and phenolic content. Furthermore, the cinnamon treatment resulted in significantly lower mean values for both variables compared to the chitosan and chitosan/cinnamon treatments.

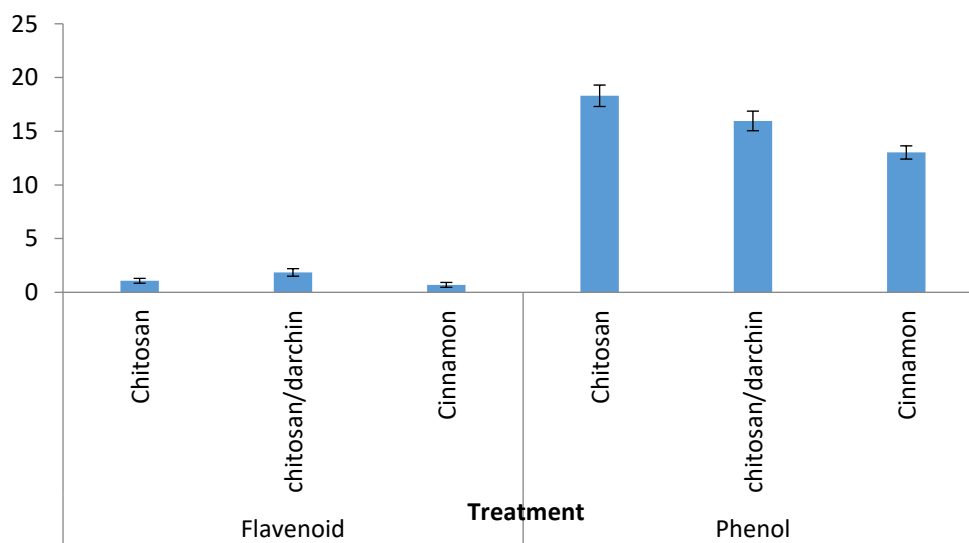


Fig. 5. Results of Duncan's multiple range test for the simple effect of treatment on the mean changes in phenol and flavonoid concentrations

3.5. Evaluation of the effect of cinnamon essential oil, chitosan, and chitosan/cinnamon treatments on the activity levels of the defensive enzymes POD and SOD

According to Table 4, the following statistical results can be extracted. Regarding the effect of the independent variables (Day and Treatment), for the variable day, the F-value was 1845.486 with a P-value much less than 0.05 ($P=0.000$), indicating that day parameter has a significant effect on the dependent variable (POD and SOD). For the variable treatment, the F-value was 184.351 with a P-value much less than 0.05 ($P=0.000$), indicating that treatment also has a significant effect on the dependent variable. Concerning the interaction between day and treatment, for the dependent variable POD, the F-value was 5.495 with a P-value of 0.001, indicating that the Day×Treatment interaction has a significant effect on POD. For the dependent variable SOD, the F-value was 14.618 with a p-value much less than 0.05 ($P=0.000$), indicating that the Day×Treatment interaction also has a significant effect on SOD. Regarding the overall effect of the independent factors, for POD, the R-squared value was 0.994 and the adjusted R-squared value was 0.992, indicating that the independent variables collectively explain approximately 99.4% of the observed variation in POD. For SOD, the R-squared value was 0.998 and the adjusted R-squared value was 0.997, indicating that the independent variables collectively explain approximately 99.8% of the observed variation in SOD. Based on the above results, it can be concluded that the independent variables (Day and Treatment) and their interaction have a significant effect on the dependent variables (POD and SOD). Furthermore, the independent variables collectively explain approximately 99.4% (for POD) and 99.8% (for SOD) of the observed variation in the dependent variables.

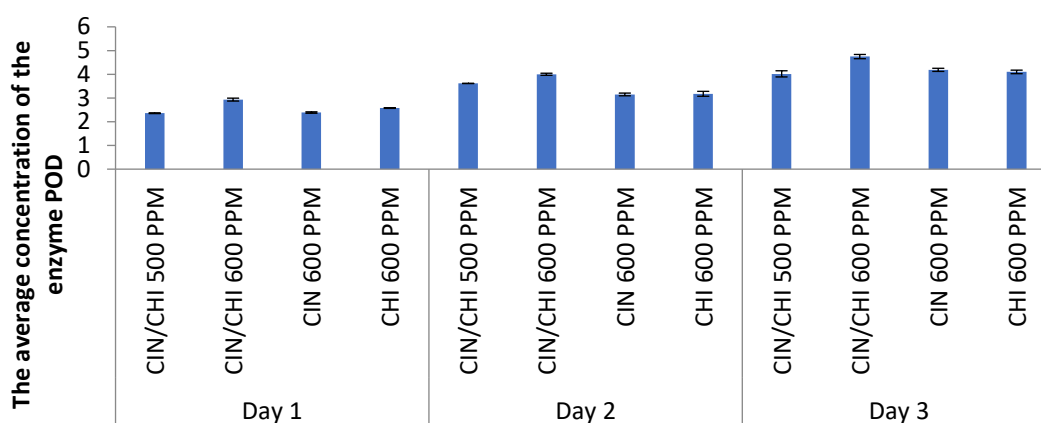
Table 4. ANOVA for the effect of cinnamon essential oil, chitosan, and chitosan/cinnamon treatments on the activity levels of the defensive enzymes POD and SOD

Tests of Between-Subjects Effects						
Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	POD	20.102 ^a	11	1.827	388.818	.000
	SOD	44.082 ^b	11	4.007	1064.719	.000
Intercept	POD	414.801	1	414.801	88255.556	.000
	SOD	874.089	1	874.089	232230.348	.000
Day	POD	17.348	2	8.674	1845.486	.000
	SOD	43.466	2	21.733	5774.017	.000
Treatment	POD	2.599	3	.866	184.351	.000
	SOD	.287	3	.096	25.390	.000
Day * Treatment	POD	.155	6	.026	5.495	.001
	SOD	.330	6	.055	14.618	.000
Error	POD	.113	24	.005		
	SOD	.090	24	.004		
Total	POD	435.016	36			
	SOD	918.262	36			
Corrected Total	POD	20.215	35			
	SOD	44.173	35			

a. R Squared = .994 (Adjusted R Squared = .992)

b. R Squared = .998 (Adjusted R Squared = .997)

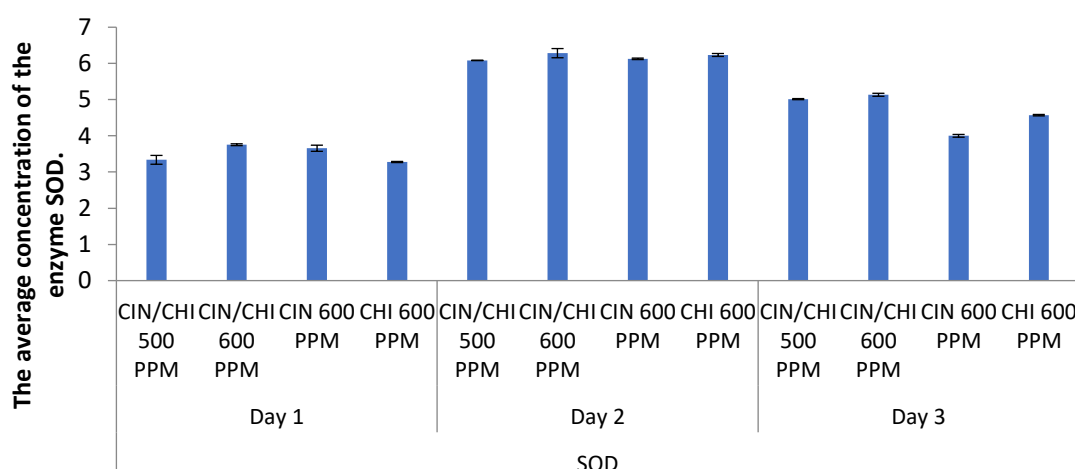
By observing the mean values of the POD enzyme, it can be concluded that different treatments have varying effects on stimulating POD enzyme activity across different days (Fig. 6). On day 1, the cin/chi 600 ppm treatment exhibited the greatest effect on POD enzyme activity, with a high mean value of 2.93. On day 2, the same treatment (cin/chi 600 ppm) again showed the highest POD enzyme value, with a mean of 4.00. On day 3, the cin/chi 600 ppm treatment continued to demonstrate the greatest effect on POD enzyme activity, with a mean of 4.75. Overall, it is observed that the cin/chi 600 ppm treatment had the greatest effect on mean POD enzyme values across all three days. This result may indicate that increasing the concentration of cin and chi to 600 ppm in the treatment leads to greater stimulation of POD enzyme activity and can produce a significant improvement in POD enzyme performance.



The interaction effect of treatment and day.

Fig. 6. Interaction plot of treatment and time on mean POD enzyme activity

By observing the mean values of the SOD enzyme, it can be concluded that different treatments have varying effects on stimulating SOD enzyme activity across different days (Fig. 7). On day 1, the mean SOD enzyme activity for the cin/chi 600 ppm and cin 600 ppm treatments was higher than that of the other treatments. On day 2, the cin/chi 600 ppm treatment exhibited the greatest effect on SOD enzyme activity, with a high mean value of 6.28. On day 3, the cin/chi 600 ppm treatment again demonstrated the greatest effect on SOD enzyme activity, with a mean of 5.17. Overall, it is observed that the cin/chi 600 ppm treatment had the greatest effect on mean SOD enzyme values across all three days.



The interaction effect of treatment and time.

Fig. 7. Interaction plot of treatment and time on mean SOD enzyme activity

4. CONCLUSION

Based on the analysis of inhibitory effects and ANOVA, it can be concluded that the positive control, 500 ppm cin/chi, and chitosan 600 ppm treatments are the most effective for inhibiting the growth of the fungus *Alternaria solani*. Furthermore, the combined treatment of 600 ppm cinnamon and chitosan (designated 600 ppm cin/chi) represents the most effective approach for reducing disease severity. The various treatments of cinnamon essential oil, chitosan, and their combination also significantly influenced the activity of the defense-related enzymes including POD and SOD. Among these, the cin/chi 600 ppm treatment exhibited the greatest effect on defense enzyme activity over the three-day evaluation period. Therefore, the application of the cin/chi 600 ppm treatment, incorporating both cinnamon and chitosan, can serve as an effective strategy for enhancing POD and SOD defense enzyme activity in the studied samples.

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AUTHOR CONTRIBUTIONS

alaa Mohammed Khalil: Conceptualization, Investigation.

Mazin Saleem Shakir: Methodology, Laboratory Analysis.

Hussein Ali Al-Bahrani: Data Curation, Formal Analysis.

Zeyad Kadhim Olewi: Supervision, Writing—Review & Editing.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICS STATEMENT

Lorem ipsum dolor sit amet, consectetur adipiscing elit. Ethical approval was not required for this study.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding author upon reasonable request

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