

Impact of Different Sources of N-Fertilizers on Growth and Nitrate Uptake in Edible Parts of Spinach under Wastewater Irrigation

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Abstract: Wastewater irrigation is increasingly used to meet the rising demand for food production, particularly for vegetables. However, residential wastewater and inorganic nitrogen fertilizers may increase nitrate accumulation in leafy vegetables, creating potential public health concerns.

Objective: To evaluate the effect of different nitrogen fertilizers on nitrate accumulation, biomass yield, and health risk index in spinach irrigated with wastewater and underground water. **Methods:** A pot experiment was conducted using a completely randomized factorial design. Four treatment combinations were tested: urea + underground water, urea + wastewater, calcium ammonium nitrate + underground water, and calcium ammonium nitrate + wastewater, with three replications each. Two spinach varieties were included: desi white and hybrid. Edible spinach biomass yield, nitrate uptake, and health risk index were assessed under each treatment combination to determine the impact of fertilizer source and irrigation water quality.

Results: The highest biomass yield and nitrate uptake were observed in spinach treated with calcium ammonium nitrate under wastewater irrigation, followed by urea + wastewater, calcium ammonium nitrate + underground water, and urea + underground water. The combined use of synthetic nitrogen fertilizers and wastewater irrigation resulted in a health risk index greater than 1, indicating a potential health concern. In contrast, spinach irrigated with groundwater showed a health risk index below 1, suggesting a comparatively lower risk. **Conclusion:** Wastewater irrigation combined with synthetic nitrogen fertilizers, particularly calcium ammonium nitrate, increased spinach biomass but also markedly enhanced nitrate accumulation and dietary health risk. These findings suggest that although wastewater may improve crop productivity, its use with inorganic nitrogen fertilizers in spinach cultivation should be carefully managed to reduce nitrate-related health hazards.

Keywords: Calcium Ammonium Nitrate; Nitrates; Spinacia oleracea; Wastewater; Watering

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Introduction

Vegetables play an important role in the human diet due to their high nutritional value. They are beneficial to the physiological functions of the human body as they are rich in sugar, starch, protein, fat, and water (1). Green leafy vegetables, including spinach, parsley, and cabbage, are considered an exceptional source of vitamins, minerals, and organic compounds (2). Spinach (*Spinacia oleracea*) is one of the dark green leafy vegetables that belongs to the family Amaranthaceae. It is native to Southwest Asia and 1st time cultivated in Persia (Iran). It comprises highly active antioxidants that are beneficial in combating cancer and diabetes, Vitamin K1 for blood clotting, and nitrate to normalize blood pressure (3).

Intensive agricultural practices (application timing and doses of synthetic N-fertilizers and wastewater irrigation) are being adopted to grow vegetables in Pakistan, and the implementation of such practices, coupled with other factors (soil texture, temperature, environmental conditions), is a significant reason for higher nitrate concentrations in leafy vegetables (4). The nitrate concentration varies across different parts of plants, as it also depends on the cultivation time. Spinach contains nitrate content up to 1000 to 3500 mg per kg of fresh weight. An adult weighing 60 kg can consume up to 211 mg/day of nitrate (5).

Untreated wastewater irrigation has become the most common practice due to freshwater scarcity, population growth, urbanization, and higher nitrate concentrations. Wastewater irrigation has been associated with

several positive and negative effects (6). Wastewater contains nitrate, organic matter, chemical substances, and often pathogens, which cause many environmental issues (7). It degrades soil quality and ultimately human health. Wastewater irrigation leads to the accumulation of heavy metals and nitrates in leafy vegetables, posing a major health concern (8). The excessive uptake of nitrates in leafy vegetables has become a serious threat to human health (9). Methaemoglobinaemia (blue baby syndrome), stomach cancer, childhood diabetes, high blood pressure, respiratory tract infection, thyroid disease (because of inhibition of iodine), and many other health-related issues occur due to the ingestion of high levels of nitrate and nitrite-based vegetables (10).

Different sources of nitrogen fertilizers (urea, ammonium sulphate, ammonium nitrate, and calcium ammonium nitrate) affect spinach yield and nitrate accumulation in different ways; for example, urea contains higher nitrogen content than ammonium nitrate and calcium ammonium nitrate (11). Calcium ammonium nitrate dissociates into ammonium and nitrate ions in soil and undergoes ionization. Urea releases the ammonia and nitrate in two steps. Firstly, urea hydrolysis occurs, in which the urease enzyme hydrolyzes urea, and the ammonia is then oxidized to nitrite by ammonia-oxidizing bacteria. After that, the nitrite oxidizes into nitrate by the nitrite-oxidizing bacteria (12).

The main focus of the current study is to examine the combined effect of synthetic nitrogenous fertilizers and untreated wastewater on growth and nitrate uptake in spinach. The wastewater application may increase nitrate uptake and pose a threat to human health. A study was thus carried out to



assess the interactive effects of nitrogenous fertilizers and wastewater on spinach growth, including nitrate uptake patterns.

Methodology

A pot experiment was carried out using two spinach varieties (Desi white and Hybrid) as test crops in the farm area of the Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad. The experiment was designed to assess the effects of different chemical N-fertilizers and irrigation sources on growth and nitrate uptake in spinach's edible parts. The soil was taken from the farm area of the Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad. The experiment consisted of 4 treatments (T1: urea +underground water, T2: urea + wastewater, T3; CAN (calcium ammonium nitrate) + underground water, T4: CAN+wastewater) with 3 replications. These treatments were applied to 2 spinach varieties (V1 was desi white and V2 was hybrid).

After the soil collection, it was dried and passed through a 2mm sieve to mix it thoroughly. The soil texture was sandy clay loam, with a pH range of 7.2 to 7.48 and an ECe of 1.65 dS m-1. Each pot was filled with 10kg of soil. A completely randomized design (CRD) with 3 replications was used as the experimental design. A basal dose of NPK fertilizer was applied at a rate of 60:50:50 kg ha⁻¹ to each pot. Nitrogen (60 kg ha⁻¹), phosphorus (50 kg ha⁻¹), and potassium (50 kg ha⁻¹) were uniformly applied as urea, CAN (calcium ammonium nitrate), Diammonium phosphate, and Murate of potash, respectively.

Agronomic Parameters and Nitrate Analysis

All the agronomic parameters (shoot fresh weight (g), root fresh weight (g), shoot and root length in cm) were recorded by using a measuring scale. The physiological parameter (chlorophyll content) was measured using a SPAD meter before harvesting. Spinach was harvested when plants had multiple leaves and were dark green. Shoot and root dry weight (g) was recorded after being oven dried at 70°C. Nitrate uptake was measured (mg/kg) using a spectrophotometer (Cataldo et al., 1975).

For vegetables, weigh 100mg of dried ground samples in a tube. Then add 5 ml of hot deionized water (90-95°C), mix thoroughly, and again add 5 ml of hot deionized water. The suspensions were incubated at 45°C for 1 hour. After mixing, centrifuge the open tubes at high speed (5000 × g) to sediment the tissue residues. The supernatants were decanted and saved for analysis to determine the nitrate absorbance at 410nm using a spectrophotometer.

Expected Daily Intake of Nitrate

Nitrate enters the human body through vegetables and fruits, but it may be fatal if its concentration exceeds the toxic level (ADI limit). According to JECFA and SCF, the limit of acceptable daily intake of nitrate is about 3.7mg/kg body weight per day. The estimated daily intake (EDI) of nitrate was calculated in order to estimate the average nitrate intake per kg body

weight per day in a person. According to WHO and FAO, daily consumption of vegetables should be 400g/day for adults (65.4kg b.wt for women and 78.1kg for men) and children (16kg). The EDI was calculated by using the following formula;

$$EDI = \frac{CL \times AF \times CR \times F \times ED}{B. \text{ weight} \times AT}$$

AT = ED × days year⁻¹(365 days).

Where:

CL = Concentration of contaminants (mg g⁻¹)

AF=bioavailability factor (1)

CR = duration of exposure that should be in years (g day⁻¹)

F = frequency of exposure (365 days).

Other characteristics were set as the bioavailability factor (AF) = 1 (meaning 100% consumption of nitrate) and exposure duration (ED) at 30 years for adults and 6 years for children.

After that, the health rate index was calculated to estimate nitrate accumulation in vegetables and fruits, indicating whether human consumption of nitrate-containing food poses a potential risk to their health. The health risk index depends on the estimated daily intake of food (EDI) and Rfd, the oral reference dose. According to USEPA (2007), the oral reference dose for nitrate is about 1.60 mg/kg body weight/day. If the HRI value is >1, it means the consumer is at potential health risk. The health risk index was calculated by the following formula;

$$HRI = EDI / Rfd$$

Statistical Analysis

ANOVA (analysis of variance) methods were used to statistically evaluate the acquired data according to the CRD (2-Factorial). At p = 0.05, the least significant difference (LSD) test was used to compare means (Steel & Torrie, 1980). For statistical analysis, the software program Statistics 8.1 was employed.

Results

All agronomic parameters (root and shoot dry weight, leaf area, and height) were affected significantly by the imposed treatments (Table 1 and 1.1). The interaction between varieties (Desi white and Hybrid) and treatments affected spinach growth parameters. In the 1st and 2nd picking, the highest dry matter production, leaf area, and shoot height were observed by the application of CAN with wastewater irrigation (T4) as compared to the application of synthetic N-fertilizers with underground water. The overall biomass of the 1st picking was relatively higher than that of the 2nd picking. The application of CAN+WW increased the dry matter by 41-48%, shoot weight by 55-57%, and leaf area by 78-85% in both V1 (desi white) and V2 (Hybrid) varieties of spinach.

Table 1: Impact of different Chemical-N fertilizers on growth parameters of spinach irrigated with wastewater

Treatments	Shoot dry weight (g)	Shoot length (cm)	Leaf area (cm)	Root length (cm)	Root fr.wt (g)	Root dry weight (g)
T1V1	3.94 h	20.63 g	6.76 d	13.66 g	5e	1.02 f
T2V1	7.5 d	30.47 d	7.4 bc	23.7 d	7.93 b	3.21 c
T3V1	5.3 f	24.06 f	7.03 cd	18.27 e	6.41 cd	1.6 e
T4V1	9.50 b	35.80 b	8.03 b	27.067 b	8.91 b	4.69 b
T1V2	4.6 g	22 g	6.83 d	15.46 f	5.63 de	1.54 e
T2V2	8.23 c	33.06 c	8 b	24.933 c	8.52 b	3.51 c
T3V2	6.18 e	26.93 e	7.26 cd	19.05 e	6.9 c	2.5 d
T4V2	10.91 a	40.46 a	8.76 a	31.5 a	10.63a	5.06 a

In each column, similar letters indicate non-significant differences. In contrast, different letters indicate significant differences between the values according to the least squares means at the 5% probability level, and LSD-separate means.

T1=Urea + underground water; T2=Urea + wastewater; T3=CAN+ underground water; T4=CAN + wastewater

V1=Desi white variety; V2= Hybrid variety of spinach

In each column, similar letters indicate non-significant differences. In contrast, different letters indicate significant differences between the values according to the least squares means at the 5% probability level, and LSD-separate means.

T1=Urea + underground water; T2=Urea + wastewater; T3=CAN+ underground water; T4=CAN + wastewater

V1=Desi white variety; V2= Hybrid variety of spinach

Impact of different Chemical-N fertilizers and wastewater irrigation on Nitrate accumulation (mg/kg) in spinach. The results indicate that the application of synthetic nitrogenous fertilizers with wastewater irrigation increased the nitrate concentration in both spinach varieties (desi white and hybrid). The highest nitrate uptake was observed with CAN application under wastewater irrigation in the V2 variety of spinach, compared to V1 (Table 2). The least level of nitrate uptake was obtained by the application of synthetic fertilizer (urea) with groundwater irrigation in both varieties (V1 and V2) of spinach. Nitrate uptake in both desi white

and hybrid varieties of spinach was relatively similar in pots with synthetic fertilizers (urea, CAN) and under groundwater irrigation. However, the application of calcium ammonium nitrate with groundwater irrigation slightly increased nitrate uptake in V2 compared with V1. The maximum nitrate uptake was observed at 43.7 mg/kg with CAN application under wastewater irrigation, and the minimum at 8.08 mg/kg with urea application under groundwater irrigation. The nitrate concentration significantly increased to 4.61mg/kg by the application of urea with wastewater irrigation in the V1 variety of spinach, and the lowest mean value of nitrate concentration was observed by the application of (urea, CAN) with groundwater irrigation.

Table 1.1: Impact of different Chemical-N fertilizers on growth parameters of spinach under wastewater Irrigation (2nd Picking)

Treatments	Shoot dry. Wt (g)	Shoot Length (cm)	Leaf Area (cm)
T1V1	2.5 f	13.667 f	3.03 h
T2V1	6.1 c	21.56 c	6.14 d
T3V1	3.8 e	17.2 de	4.53 f
T4V1	7.51 b	30.43 a	7.53 b
T1V2	3.3 e	15.88 e	3.9 g
T2V2	6.68 c	23.5 b	6.81 c
T3V2	5.3 d	17.5 d	5.16 e
T4V2	9.103 a	31.46 a	9.23 a

Table 2: Impact of different Chemical-N fertilizers on nitrate concentration. and nitrate uptake of spinach under wastewater Irrigation

Treatments	1 st Picking			2 nd Picking		
	NO ₃ conc. mg/kg dry weight	ADI mg/kg B.wt	NO ₃ uptake mg/kg dry.wt	NO ₃ conc. mg/kg dry.wt	ADI mg/kg B.wt	NO ₃ uptake mg/kg dry.wt
T1V1	2.23 e	3.7	8.08 d	2.08 c	3.7	5.2 d
T2V1	4.61 a	3.7	34.57 c	4.41 a	3.7	26.84 c
T3V1	1.61 e	3.7	8.34 d	1.49 d	3.7	5.66 d
T4V1	4.18 bc	3.7	39.71 b	4.1b	3.7	30.75 b
T1V2	1.91 d	3.7	8.53 d	1.68 d	3.7	5.54 d
T2V2	4.3 b	3.7	35.38 c	4.2 ab	3.7	28 c
T3V2	1.5 e	3.7	9.27 d	1.09 e	3.7	5.7 d
T4V2	4.01c	3.7	43.70 a	4 b	3.7	36.4 a

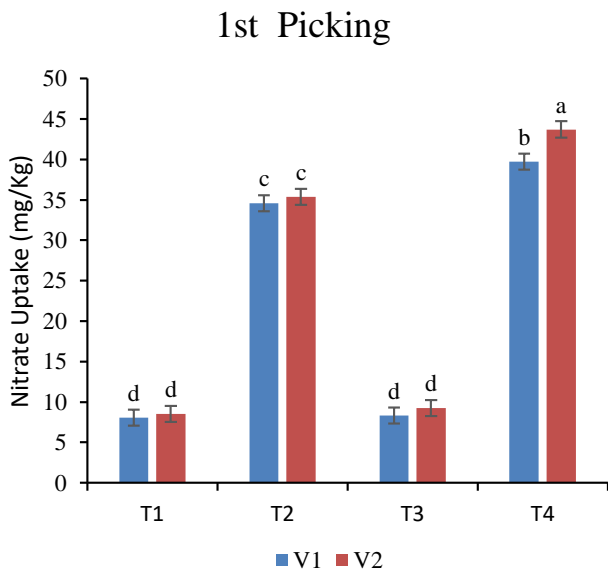


Figure:1

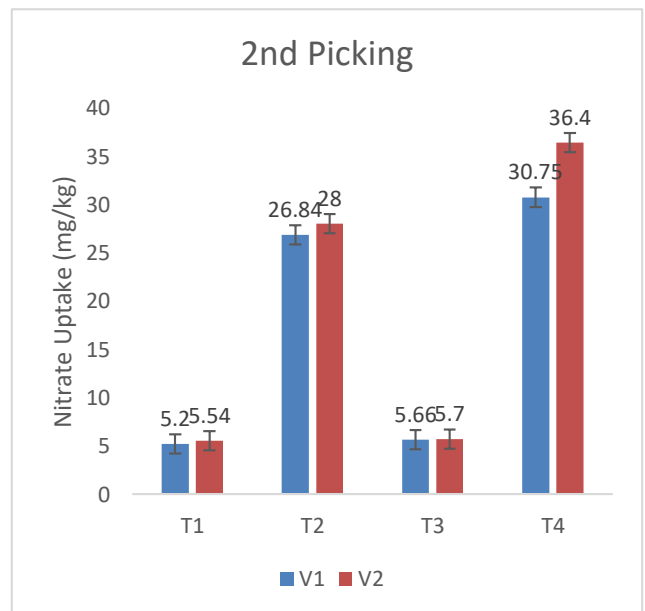


Figure:2

Table 3. Expected daily intake (EDI) and hazard quotient (HQ) for spinach under wastewater irrigation according to the recommendations of WHO and FAO for children and adults (1st Picking).

Treatments	According to the WHO recommendation of consumption (400g/day) (1st Picking)					
	ED1 (mg NO3/Kg B.wt/Day)			HQ		
	women	men	Children	Women	Men	Children
T1V1	0.89	0.89	0.89	0.55	0.55	0.55
T2V1	1.84	1.84	1.84	1.15	1.15	1.15
T3V1	0.64	0.64	0.64	0.40	0.40	0.40
T4V1	1.67	1.67	1.67	1.04	1.04	1.04
T1V2	0.72	0.72	0.72	0.45	0.45	0.45
T2V2	1.72	1.72	1.72	1.07	1.07	1.07
T3V2	0.6	0.6	0.6	0.37	0.37	0.37
T4V2	1.60	1.60	1.60	1.00	1.00	1.00

Table 3.1 Expected daily intake (EDI) and hazard quotient (HQ) for spinach under wastewater irrigation according to the recommendations of WHO and FAO for children and adults (2nd Picking).

Treatments	According to the WHO recommendation of consumption (400g/day) (2 nd Picking)					
	ED1 (mg NO3/Kg B.wt/Day)			HQ		
	women	men	Children	Women	Men	Children
T1V1	0.832	0.832	0.832	0.52	0.52	0.52
T2V1	1.76	1.76	1.76	1.1	1.1	1.1
T3V1	0.59	0.59	0.59	0.36	0.36	0.36
T4V1	1.64	1.64	1.64	1.02	1.02	1.02
T1V2	0.67	0.67	0.67	0.41	0.41	0.41
T2V2	1.68	1.68	1.68	1.05	1.05	1.05
T3V2	0.43	0.43	0.43	0.26	0.26	0.26
T4V2	1.6	1.6	1.6	1	1	1

T1=Urea + underground water; T2=Urea + wastewater; T3=CAN+ underground water; T4=CAN + wastewater V1=Desi white variety; V2= Hybrid variety of spinach

Discussion

Nitrogen is an essential nutrient for plant growth, especially in alkaline or calcareous soils (13). It is one of the major nutrients that plants need to regulate their growth and Development. It is important for plants to flourish and to provide strength. It is the most significant component of chlorophyll that gives plants their green color and makes food through photosynthesis (absorbs sunlight and converts it into carbohydrates) (14). Therefore, the application of inorganic nitrogenous fertilizers (Urea, Calcium ammonium nitrate) is required to address nutrient deficiencies and improve leafy vegetable growth (15). The nutrient use efficiency of nitrogenous fertilizers depends upon the type of fertilizer, soil conditions, time, and method of application (16). The application of municipal and industrial wastewater has now become the most common practice in worldwide agriculture to meet water needs arising from freshwater scarcity, population growth, urbanization, and economic development (17).

Wastewater irrigation has been associated with both positive and negative effects. WWd is a rich source of nutrients that contain a desirable amount of potassium, phosphorus, and nitrogen for the growth of leafy vegetables (7). The farmers' need to use expensive chemical fertilizers is reduced for the growth of leafy vegetables because of WWd application, which is 9-20% cost-effective in agriculture (18,19). Using wastewater for vegetable production is beneficial in water conservation and in those areas where water scarcity is a major issue (20).

Wastewater irrigation is cost-effective in vegetable production, but it has some drawbacks because it increases the potential health risk by contaminating the leafy vegetables with toxic substances. Wastewater consists of harmful contaminants, including heavy metals (Pb, Cd, Cr), nitrate, and other toxic substances (21). Vegetables absorb these toxic substances through their roots and accumulate them in their stems and leaves, posing a human health risk (22).

Plant biomass has been increased by the application of CAN+ WW due to the presence of fast-acting (ammonium) and slow-releasing (nitrate) nitrogen, making CAN a better product with a neutral pH than urea. It ensures the plants receive a continuous supply of nitrogen over time, even in the soil with varying nutrient availability due to wastewater irrigation (23). A significant positive effect of chemical fertilizer on vegetable production and wastewater irrigation has been reported by Kaymak (11), Hutchinson et al. (24), and Kauser et al. (25). The highest nitrate uptake was also observed with CAN under wastewater irrigation, due to high biomass production. The results are similar to those of Kaymak (11), who reported that the application of calcium ammonium nitrate significantly increased nitrate uptake compared to other synthetic nitrogenous fertilizers (urea, ammonium nitrate, and ammonium sulphate).

The nitrate concentration varies across leafy vegetables when using different combinations of inorganic nitrogenous fertilizers (urea, CAN) with wastewater and underground water irrigation. The highest nitrate concentration was observed in Urea+ WW as compared to CAN because urea contains a high concentration of nitrogen (46%), which must be converted into ammonium and then into nitrate by soil bacteria through a process called nitrification before plants can take up. In wastewater-irrigated soil, CAN is most readily taken up by plants, resulting in lower nitrate concentrations in plant tissues than with urea (12). Xavier et al. (26) reported that lettuce grown with wastewater irrigation had a higher nitrate content. Wastewater is a rich source of N (80%), P (50%), and K (70%). The nitrate concentration significantly increased with urea application under wastewater irrigation in the V1 spinach variety. Zhou et al. (28) observed that nitrate concentration in leaves of Cole crops (Broccoli, Cauliflower, Cabbage, Kale) with different nitrogenous fertilizers varied in the order: urea > ammonium carbonate > ammonium nitrate > ammonium sulphate, whereas in petioles it varied in the order: urea > ammonium nitrate > ammonium sulphate > ammonium carbonate. The hazard quotient factor (HQ) increased up to 1 by applying both nitrogenous fertilizers (urea, CAN) with wastewater irrigation. If the HQ

value is < 1 or > 1 , it indicates a potential health risk. The consumption of such vegetables is not safe for the consumer's health. Nawaz et al. (29) and Zavadil (30) reported that high nitrate concentrations and HQ factors (> 1) were observed under wastewater irrigation. The HQ value for 1st picking was relatively close to that for 2nd picking.

The fresh biomass of leafy vegetables has decreased significantly in the 2nd picking across all treatments due to regrowth limitation and nutrient depletion. The same result was reported by Sophea et al. (31), who found that fresh biomass was significantly reduced in the 2nd harvest when urea and bio-digester effluents were applied.

Conclusion

The application of both synthetic nitrogenous fertilizers (urea, CAN) with wastewater irrigation had a positive impact on the spinach growth. Among all treatments, the application of CAN with wastewater irrigation exhibits the highest phenotypic traits, including shoot dry weight (10.91g), shoot length (40.46cm), leaf area (8.76 cm²), and root development, thereby enhancing vegetative growth in the hybrid variety (V2) of spinach. It also increased nitrate accumulation (43.70 mg/kg) and the hazard quotient (> 1) of spinach, posing a potential health risk to consumers, compared with its application under underground water irrigation (HQ < 1). The application of CAN with underground water shows moderate spinach growth, with the lowest nitrate accumulation and an acceptable health risk value according to the WHO/FAO safe limit. So, it is preferable to apply synthetic fertilizers underground to mitigate the health risks associated with elevated nitrate accumulation and the hazard quotient in spinach.

Declarations

Data Availability statement

All data generated or analysed during the study are included in the manuscript.

Ethics approval and consent to participate

Approved by the department concerned. (IRBEC--03-24)

Consent for publication

Approved

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Conflict of interest

The authors declared no conflict of interest.

Author Contribution

SF, FS, NUHZ

Contributed to study design, data collection, and initial manuscript drafting

Assisted in data acquisition, literature review, and manuscript editing
Performed statistical analysis and contributed to the interpretation of results

Helped in methodology development, data organization, and manuscript formatting

Contributed to patient recruitment, data entry, and results compilation

RB, SF, AI, AG

Assisted in referencing, proofreading, and final revisions of the manuscript

Guided study execution and critically reviewed the manuscript

Supervised the research, coordinated among authors, finalized the manuscript, and approved the final version

All authors reviewed the results and approved the final version of the manuscript. They are also accountable for the study's integrity.

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