

Antibacterial Activity of *Heliotropium crispum* Desf., a Herb from Cholistan Desert, Bahawalpur, Pakistan

Muhammad Nasir Younis¹, Arslan Saleem Chughtai², Muhammad Qasim Hayat¹

ABSTRACT

Background

This global dependency on the use of plant-derived pharmacological and chemical constituents with antibacterial properties necessitates the development of strategies to counter antibiotic resistance, which remains one of the most important issues in infection control. With this background, *Heliotropium crispum* Desf. has been identified as a prospective candidate for the development of alternative antibacterial agents.

Objective

The objectives of this study were to evaluate the antibacterial properties of the whole-plant extracts of *Heliotropium crispum* Desf. against various bacterial strains. The bacterial strains tested were *Enterococcus*, *Serratia marcescens*, *Acinetobacter*, and *Staphylococcus aureus*.

Methods

The antibacterial activity of the *Heliotropium crispum* Desf. extracts was tested using the whole-plant powder extract of *Heliotropium crispum* Desf. in four different solvents: methanol, ethanol, chloroform, and aqueous. The antibacterial activity of the *Heliotropium crispum* Desf. extracts was tested using the disc diffusion method.

Results

The study found that the *Heliotropium crispum* Desf. zones of inhibition were observed against *S. marcescens* but these did not reach statistical significance across concentrations. However, none of the *Heliotropium crispum* Desf. extracts showed any antibacterial activity against the other

bacterial strains tested: *Enterococcus faecalis*, *Acinetobacter junii*, and *Staphylococcus aureus*. The study also tested various concentrations (25 mg/mL, 50 mg/mL, 100 mg/mL, 150 mg/mL, 200 mg/mL) of the three solvents: methanol, ethanol, and aqueous. All the solvents tested had antibacterial activity against *Serratia marcescens*. However, the chloroform solvent failed to show antibacterial activity against *Serratia marcescens*.

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Conclusion:

The antibacterial properties of the *Heliotropium crispum* Desf. extracts suggest that this plant may be developed into medicinal agents.

Key Words

Medicinal Plants, *Heliotropium crispum* Desf., *Enterococcus faecalis*, *Serratia marcescens*, *Acinetobacter*, *Staphylococcus aureus*, Anti-Bacterial Agents, Microbial Sensitivity Tests

INTRODUCTION

The scope of the knowledge base of medicinal plants is huge, with healers relying on different species of medicinal plants to cure different diseases, ranging from inflamed conditions, cough and cold, skin infections, ulcers, leprosy, rheumatism, fever, dysentery, and bites of venomous snakes, among others [1].

The investigations done by ethnobotanists in different places reveal the use of different medicinal plants by healers of different places, which not only helps in the preservation of traditional knowledge but also has significant potential to help discover new medicines [1].

Heliotropium crispum Desf., a plant belonging to the Boraginaceae family and the *Heliotropium* genus, is a plant with medicinal importance that has been traditionally used for treating different diseases [2]. Abundant in bioactive compounds, *Heliotropiumcrispum* consists of pyrrolizidine alkaloids, terpenoids, and flavonoids. Thus, it has great

pharmacological importance, which requires more investigation into its ethnobotany [3].

In conventional medicine, *Heliotropium crispum* has been used in the treatment of wounds, inflammation, and other medical applications, indicating that the presence of bioactive compounds with possible medicinal properties is not out of question [4]. Due to its significance in ethnomedicine despite the lack of scientific research in this regard, the potential antibacterial properties of this plant were considered worthy of investigation to scientifically validate its efficacy [5].

Another reason for the selection of *Heliotropium crispum* is due to the plant's indigenous nature in Pakistan and significance in the region in the realm of conventional medicine. Researching indigenous plants with medicinal applications can be considered another approach for discovering new alternatives of antimicrobial compounds, especially considering the growing issue of resistant pathogenic bacteria [6].



Lastly, due to the presence of other reports in the literature regarding antimicrobial activities of other *Heliotropium* members, this particular plant was also considered scientifically worthy to test in relation to its antimicrobial potential. The reasons being the scarcity and lack of consistency in previously published literature regarding this species specifically.

Enterococcus faecalis is known for its presence in the human gut and the hospital setting due to resistance to penicillins and cephalosporins; this increases the risks of hospital-acquired infections [7]. Similarly, *Serratia marcescens*, which is a known causative agent of nosocomial infections, has also been known for resistance to many antimicrobial drugs. It further proves the ongoing struggle against antibiotic resistance [8]. *Acinetobacter junii* is not a known pathogen but is showing a prompt increase in resistance to antimicrobial drugs, which indicates the need to remain vigilant and take stringent prevention measures while dealing with nosocomial acquired infections [9]. *S. aureus* is one of the key causative agents for nosocomial acquired infections; the prevalence is significant, and the resistance pattern is also very significant for the ongoing struggle to be taken against this serious problem. *S. aureus* needs constant vigilance to oversee the development of newer treatment

options for the infections it causes [10-12]. Multidrug-resistant *Serratia marcescens* has now emerged as a serious problem; hence, the ongoing struggle to understand the antibacterial activity is more prominent to drive ways to overcome ongoing Infections [13].

The objective of this study was to evaluate the antibacterial activity of methanol, ethanol, chloroform, and aqueous extracts of *Heliotropium crispum* Desf. against clinically relevant bacterial strains (*Enterococcus faecalis*, *Serratia marcescens*, *Acinetobacter junii*, and *Staphylococcus aureus*).

METHODS

Study Design and Setting

The proposed research work was an Experimental In-Vitro Study carried out over a duration of 1 year, starting in July 2013 and ending in June 2014, in the Medicinal Plant Laboratory, Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST), Islamabad, Pakistan. The main aim was to determine the antibacterial potential of *Heliotropium crispum* Desf. extracts against the clinically important bacterial species, namely *Enterococcus faecalis* (IARS1), *Serratia marcescens* (IARS6), *Acinetobacter junii* (IARS2), and *Staphylococcus aureus* (IARS7).



Inclusion Criteria for Bacterial Strains

- Strains of bacteria frequently found to cause infections or have clinical significance.
- Pure and confirmed bacterial strains available from a reputed source or culture center.
- Gram-positive and Gram-negative bacterial strains included to assess antibacterial activity in a wider range.
- Culturable strains of bacteria that can be grown using standard laboratory practices.
- Strains having any significance regarding antimicrobial resistance or health issues.
- Strains that have stable growth for reproducibility of tests.
- Availability of standard reference strains or clinical strains of bacteria.

Exclusion Criteria for Bacterial Strains

- Mixed or contaminated cultures of bacteria.
- Bacteria with unsteady growth characteristics.
- Bacterial strains with laboratory results that vary significantly.
- Environmental bacteria that pose no threat to health and are unrelated to the purpose of the research.
- Multiple strains that exhibit similar traits and offer no scientific significance.
- Bacteria with low viability and poor growth.

- Pathogenic strains whose handling requires a biosafety level beyond what was available at the laboratory.

Bacterial Strains and Identification

Randomization of sample order and blinding of the person measuring inhibition zones were not applicable in this study. These isolates were well characterized clinically in the microbiology laboratory at Pakistan Institute of Medical Sciences (PIMS) Hospital, Islamabad, and were generously provided to ASAB, NUST, for research purposes. Among these bacteria, two Gram-positive and two Gram-negative strains were chosen. These isolates were visually identified based on colony morphology by Taxonomist Dr. Muhammad Qasim Hayat before use.

The bacterial strains that were used for this study, namely *Staphylococcus aureus*, *Enterococcus* spp., *Serratia marcescens*, and *Acinetobacter* spp., were obtained from PIMS, Islamabad.

Plant Collection and Identification

The plant voucher specimen number *Heliotropium crispum* Desf. 2321/CIDS/IUB was collected from the Cholistan Desert, Bahawalpur, and was identified by Taxonomist Dr. Muhammad Qasim Hayat.

After collection, the plant was reduced to a fine powder and used to prepare different extracts, namely aqueous, ethanol,



methanol, and chloroform. The plant extraction process involved Soxhlet extraction, rotary evaporation, centrifugation, and filtration. The prepared extracts were then diluted in 70% DMSO. DMSO was used so that there was no effect of solvents on the antibacterial activity. DMSO makes sure that the antibacterial activity was only due to the plant extracts.

Preparation of Bacterial Culture

For culture preparation, nutrient agar was formulated by dissolving beef extract, peptone, and agar at concentrations of 3 g, 5 g, and 15 g, respectively, in distilled water.

Antibacterial Activity

The antibacterial potential of *Heliotropium crispum* plant extracts was determined by employing a disc diffusion method, which included inoculating bacterial plates, applying sterilized discs, and administering dilutions of plant extracts. The antibacterial potential was quantitatively determined by measuring the inhibition zone after incubation at 37°C for a period of 20 hours. Due to time constraints and limited resources, Minimum Inhibitory Concentration was not determined.

Statistical Analysis

The Kruskal-Wallis H test was done using SPSS, which determined the significance of difference between the tested groups, with $p < 0.05$ considered statistically significant.

Study Aim

The aim of this study was to reveal the medicinal properties related to *Heliotropium crispum* plant species.

RESULTS

The antibacterial activity of the extracts was tested using the standard disc diffusion method to evaluate the effectiveness of the methanol, ethanol, chloroform, and aqueous extracts of *Heliotropium crispum*. The extracts did not show any significant antibacterial activity against *Acinetobacter junii*, *Staphylococcus Aureus*, and *Enterococcus faecalis*. However, after standardizing the activity of the extracts, the 200 mg/mL, 150 mg/mL, 100 mg/mL, 50 mg/mL, and 25 mg/mL concentrations of the extracts zones of inhibition were observed but no statistically significant concentration-dependent effect was demonstrated, with the exception of the chloroform extract, which did not show any activity at all concentrations as shown in Table 1.



Table 1. Antibacterial activity of different concentrations of *Heliotropium crispum* extracts against *Serratia marcescens*

Extract	Replicate	200 mg/mL	150 mg/mL	100 mg/mL	50 mg/mL	25 mg/mL
Methanol	1	8	10	9	9	10
	2	10	9	9	10	12
	3	9	10	9	10	11
Ethanol	1	11	7	10	8	10
	2	12	9	7	9	10
	3	8	6	9	9	9
Chloroform	1	0	0	0	0	0
	2	0	0	0	0	0
	3	0	0	0	0	0
Aqueous	1	10	9	9	10	10
	2	10	8	9	9	10
	3	9	9	9	9	10
Positive control: Amoxicillin (Amx)	—	NA	NT	5 mm	7 mm	22 mm
Positive control: Gentamicin (Gen)	—	NA	NT	20 mm	24 mm	23 mm
Negative control: DMSO	—	NA	0	0	0	0

Footnotes: Values represent zones of inhibition (mm). Abbreviations: Amx, amoxicillin; DMSO, dimethyl sulfoxide; Gen, gentamicin; NA, not applicable; NT, not tested.

The inhibition zones of the extracts ranged from 6-12 mm, indicating activity against *Serratia marcescens*. The results were compared with positive and negative control groups. All experiments were done in triplicate, with the results expressed as means with standard deviation. As the data was not normally distributed Kruskal Wallis H test was used. There was no

statistically significant difference between ‘*Serratia marcescens* Zones of Inhibition(mm) according to different extracts at 25mg/ml concentration (p-value 0.113), 50mg/ml (p-value 0.179), 100mg/ml (p-value >0.999), 150mg/ml (p-value 0.088) and 200mg/ml(p-value 0.513) Figure 1.



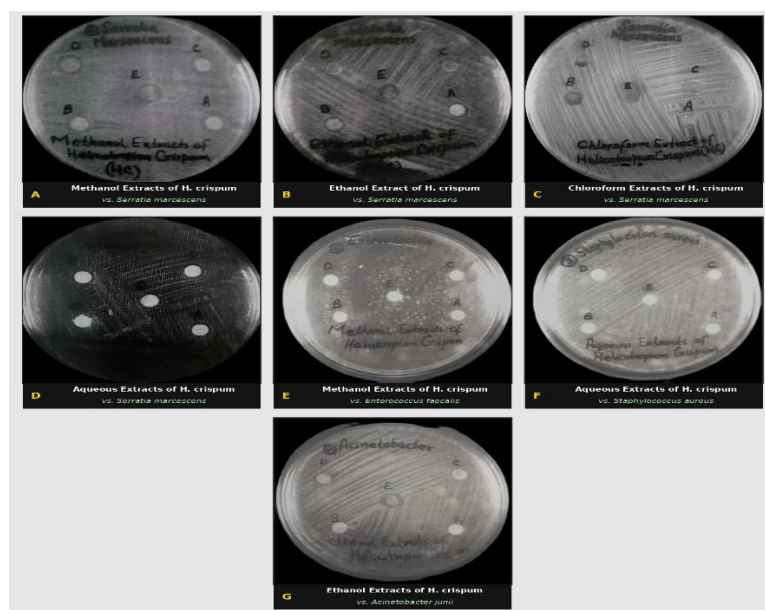


Figure 1. Antibacterial activity of *Heliotropium crispum* extract against various bacterial pathogens assessed using the Kirby–Bauer disk diffusion assay.

In methanol the difference in zone of inhibition according to concentration was not statistically significant (p-value 0.092). Similarly, in ethanol difference in zone of inhibition according to concentration was not statistically significant (p-value 0.224) and in Aqueous difference in zone of inhibition according to concentration was not statistically significant (p-value 0.061).

DISCUSSION

Heliotropium crispum Desf., a member of the Borage family, has long been known for its antidepressant and wound-healing properties, along with its diuretic activities [6]. Although some of these plants, such as *Heliotropium indicum* L., possess hepatotoxic properties in terms of alkaloid content, these are not recommended for internal administration, but nonetheless possess a variety of medicinal activities,

including antibacterial, antitumor, and anti-inflammatory properties, which are used to treat liver disorders in medicine [14].

Rashid et al.'s team did not detect antibacterial activity in the *Heliotropium crispum* Desfherb preparation [15]. However, other *Heliotropium* preparations did exhibit antibacterial properties in further studies, such as the investigation conducted by Rahimifard, where the antibacterial properties of *Heliotropiumbacciferum* herb preparation were exhibited in the inhibition of bacterial strains such as *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* [16]. Another team, documented the weak antibacterial properties of the *Heliotropiumellipticus* herb preparation, particularly in the preparation rich in alkaloid compounds

[17]. Another investigation conducted in the United Arab Emirates region documented the inconsistency in antibacterial activity in the *Euphorbia helioscopia* herb preparation extracted using the solvent on the stems [18]. This indicates that Rashid and team's study documentation did not entail antibacterial properties for the *Heliotropium crispum*. From the work of Rashid et al. Rahimifard et al. Bakht et al. and Shahin et al. antimicrobial property of plants seems to be affected by methodology rather than the taxonomy of the plant species [15-18]. While Rashid et al. indicated no or minimal antibacterial activities in *Heliotropium crispum*, this should not be considered a definitive sign of lack of antimicrobial properties. The differences in methodologies, solvent used, part of the plant used, phytochemical constituents and test organisms seem to be responsible for the differences seen in studies.

The most important point is that of the extraction procedure. In the study conducted by Rashid et al. crude ethanol, ether and aqueous extract of the plant was used. Such crude extraction is likely to result in the presence of active chemical constituents present at levels below the level of detection [15]. On the other hand, Rahimifard et al. and Bakht et al. used solvent-partitioning method to isolate

active chemical constituents like flavonoids, phenolic compounds, alkaloids and terpenoids [16,17].

Differences in species could be another explanation for the observed discrepancies. Although *Heliotropium crispum* and *H. bacciferum* are from the same genus, close relatives usually show variations in terms of phytochemistry owing to the influence of both genotype and environment [16]. Similarly, extracts obtained from different parts of plants are not expected to have equal amounts of secondary metabolites, accounting for the increased activity of *H. bacciferum*'s aerial parts and *Euphorbia helioscopia*'s stems [16,17].

Susceptibility of microorganisms to extracts should not be overlooked. Differences in cell wall and plasma membrane permeability and resistance to antibiotics mean that bacteria cannot have the same level of sensitivity to the extracts [18]. Selective toxicity of *Heliotropium crispum* to *Serratia marcescens* is explained by species-specific sensitivity, quorum sensing inhibition, differential membrane permeability, and the low concentration of antimicrobial secondary metabolites in the plant.

The specific antibacterial action of *Heliotropium crispum* against *Serratia marcescens* can be explained by the presence of phytochemicals that include

phenols, flavonoids, alkaloids, and terpenoids. Phytochemicals are recognized as having antibacterial action through various mechanisms such as the destruction of cellular membranes, inhibition of protein synthesis, quorum-sensing inhibition, and inhibition of biofilm formation. Quorum-sensing inhibition is especially significant as *Serratia marcescens* uses cell-to-cell signaling in order to regulate biofilm formation, motility, production of its distinctive red-colored prodigiosin pigment, among other processes. Most plant phenolics and flavonoids are known to inhibit bacterial pathogenicity through quorum-sensing inhibition rather than direct cell death induction. Moreover, there might be certain physicochemical characteristics of *Heliotropium crispum*'s active ingredients that allow them to easily penetrate into the cell of *S. marcescens*, circumvent any bacterial efflux systems, achieve inhibitory levels inside target cells, while having no significant activity on other types of bacteria. One more possible reason for the phenomenon might be that the concentration of active compounds in *Heliotropium crispum* is low enough to inhibit just the most susceptible strain from the group under examination, while leaving other bacteria unaffected. Moreover, different extraction conditions can have a

substantial impact on the efficiency of isolation of phytochemicals with antimicrobial potential. Solvent, extraction time, temperature, and even the maturity of the collected plants can have an effect on the concentration and structure of antimicrobial agents. It is thus very likely that the selectivity of antibacterial action exhibited by *Heliotropium crispum* extracts is associated with the above-listed reasons. It appears from the above that *Heliotropium crispum* has narrow-spectrum antimicrobial properties, while those of *H. bacciferum* and *Euphorbia helioscopia* are broader under optimal extraction conditions. Further studies are necessary to investigate the chemical nature of the compound(s) contributing to *Heliotropium crispum*'s selectivity.

The current work was aimed at testing the efficacy of the extracts of *Heliotropium crispum* Desf. with methanol, ethanol, chloroform, and water solvents to inhibit bacteria Figure 2. The panel of bacteria used in the study included two Gram-positive bacteria and two Gram-negative bacteria. Some of the findings showed that the methanol, ethanol, and water-based extracts inhibited *Serratia marcescens* with zones of inhibition that ranged from 7.33 mm to 11 mm in diameter.



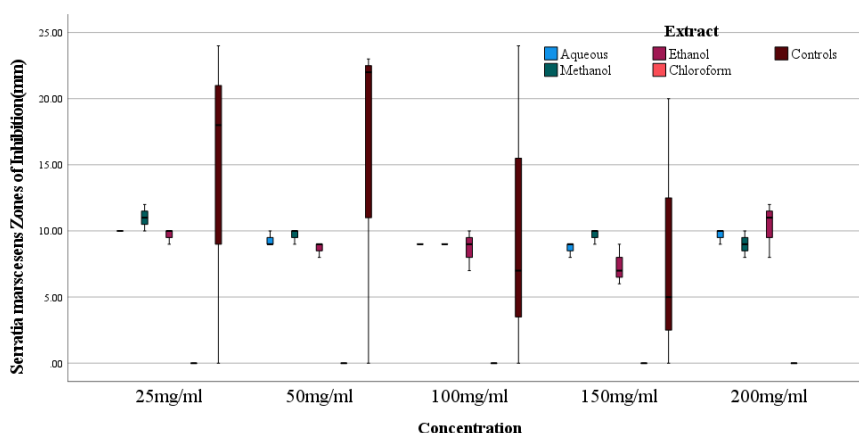


Figure 2: Comparison of efficacy of extracts at different concentrations *Heliotropium crispum* at 25mg/ml, 50mg/ml, 100mg/ml, 150mg/ml and 200mg/ml and Controls against '*Serratia marcescens*.

Another thing that *Serratia marcescens* is noted for is its pathogenic effect on common pest populations of *lepidopteran* insects, an effect that poses a very real threat to insects living in insectaries [19]. *Heliotropium crispum*, on the other hand, has been recognized as susceptible to infection with the rust fungus, *Uromyces heliotropii*, an element that has been useful in controlling heliotrope infestation, and this has formed a backdrop for an examination of *Heliotropium crispum* as a potential instrument of control over *S. marcescens* [20].

Even though zones of inhibition for methanol, ethanol, and aqueous extracts were noted against *Serratia marcescens*, no statistical difference between the concentrations was shown after application of Kruskal–Wallis test. Hence, one cannot conclude on the concentration dependence of antibacterial activity of the plant extract

against this microorganism under studied conditions.

Chloroform extract failed to show antibacterial effect against any of the tested strains of bacteria. Such an effect might be due to poor solubility of chloroform in the presence of bioactive compounds. Since chloroform is non-polar, it is likely to be incapable of dissolving polar bioactive compounds such as phenolics, flavonoids, and some alkaloids which usually demonstrate antimicrobial activity [21,22]. No antibacterial activity was recorded against *Acinetobacter junii*, *Enterococcus faecalis*, and *Staphylococcus aureus*. Divergent sensitivities of bacteria might be linked to varying structure of their cell wall, different permeability of membrane, enzymatic and efflux mechanisms of resistance [22].

The cell wall of Gram-positive bacteria is thick due to peptidoglycan layer [23]. The

latter is not typical for Gram-negative bacteria that have an outer membrane abundant in lipopolysaccharides.

The Kirby-Bauer disk diffusion test showed that gentamicin had strong antibacterial effects against all tested strains of *Serratia marcescens* with inhibition zones varying between 20-24 mm, showing high susceptibility. It is also clear that the antibacterial properties observed are in line with the effectiveness of aminoglycosides on Gram-negative bacteria. On the other hand, amoxicillin displayed inconsistent results with inhibition zones of only 5 and 7 mm against two strains due to resistance and larger zones of 18 and 22 mm against the other strains. Such inconsistency can be related to variation in β -lactamase activity among different strains of *S. marcescens*, especially AmpC β -lactamases. In summary, gentamicin was more potent than amoxicillin in its antibacterial properties. These results are critical to compare the antibacterial effect of *Heliotropium crispum* against *S. marcescens* since, for any activity shown, it is not via the mechanism of β -lactam antibiotics.

In this regard, resistance patterns exhibited during this research suggest that antibacterial activity is not determined by Gram categorization alone, because in this case, antibacterial activity was seen only against *Serratia marcescens* [24]. Results

have shown specific antibacterial action on *Serratia marcescens*; however, the possibility of further antimicrobial activities was ruled out owing to the lack of action on other strains.

LIMITATIONS

The current study had several limitations. First, the use of just four bacterial strains meant that the results could not be generalized. Second, crude plant extract was tested without the identification and purification of the phytoconstituents involved. Moreover, there was no measurement of MIC and MBC, which are very important when testing the efficacy of antibacterial drugs. What is more, although antibacterial activity against *Serratia marcescens* was noted, it was insignificant at all concentrations tested with the help of the Kruskal-Wallis test. Finally, toxicity studies were not carried out. This means that further research is needed in order to determine the medicinal value of *Heliotropium crispum* extracts.

CONCLUSION

Thus, a thorough examination and investigation of *Heliotropium crispum* Desf. indicate it has real potential in many areas. By examining the phytochemical composition of the plant, it has been noted that it possesses a number of interesting bioactive chemical constituents with antifungal, antibacterial, and antiviral



activity. By examining the interaction of the plant's constituents in relation to standard antibiotics with the help of a number of solvents, using modern techniques of biochemistry, and strict antibacterial tests, there is the potential for doors to be opened that could provide a real breakthrough. Also, toxicological examination could also help to reveal the potential use of *Heliotropium crispum* Desf. in the field of medicinal science.

DECLARATIONS

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Data Availability Statement

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

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this study.

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None.

AUTHOR CONTRIBUTIONS

Study conception and design: Muhammad Qasim Hayat.

Data collection and management: Muhammad Nasir Younis.

Data analysis: Muhammad Nasir Younis, Arslan Saleem Chughtai.

Manuscript writing and editing: Muhammad Nasir Younis.

Final approval of the version to be published:

Muhammad Nasir Younis, Arslan Saleem Chughtai, Muhammad Qasim Hayat.

All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work.



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