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## ACKNOWLEDGEMENT

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# Green Nanobiotechnology: Focusing on Plant-Mediated Synthesis of Nanoparticles as An Eco-Friendly Alternative to Traditional Chemical Methods

OPEN ACCESS

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Nanoparticles (NPs) have revolutionized biomedicine, agriculture, environmental remediation, electronics, and materials science through precise control of size, shape, and surface chemistry [1, 2]. Yet the conventional method through chemical synthesis has proven environmentally unsustainable and biologically hazardous. Chemical synthesis often involves expensive reagents, which have a detrimental effect on the environment [3]. This editorial argues that plant-mediated green synthesis is the is a more effective, sustainable, and forward-looking approach. However, green synthesis is an eco-friendly, sustainable, cost-effective, and safe method for manufacturing nanoparticles [4]. By using plant extracts as natural reducing and capping agents, this approach delivers high-quality NPs under mild, aqueous conditions while eliminating toxic reagents, energy waste, and hazardous by-products [5].

Traditional chemical synthesis depends on harsh reducing agents such as sodium borohydride, the most widely used reductant, along with organic solvents and elevated temperatures or pressures [6]. These processes generate toxic waste, require expensive disposal protocols, and leave residual chemicals in the environment. This creates long-term environmental and health hazards [7]. Comparative studies repeatedly demonstrate that organic solvents have higher health risks, including behavioral, reproductive, and neurological effects, with longer environmental persistence [8]. Sometimes, the nanoparticles synthesized using the green method have enhanced quality and size compared to chemical synthesis. A recent study investigated the comparison of synthesizing Fe304 nanoparticles using chemical and green methods. The size of NPs using the chemical method was 87-400 nm, which is much larger than the size of 2-80 nm of nanoparticles synthesized via green synthesis [9].

Plant-mediated green synthesis offers a complete contrast. It offers no harsh chemicals, is non-toxic, cost-effective, environment-friendly, sustainable, and a safe option for the synthesis of NPs. Additionally, by employing green reducing and capping agents, the NPs show unique properties such as biocompatibility and enhanced stability [10]. Plant extracts are packed with diverse phytochemicals such as polyphenols, flavonoids, alkaloids, terpenoids, proteins, and enzymes that function as powerful capping agents, reducing agents, and stabilizers. As these compounds donate electrons to metal ions while preventing aggregation and enhancing stability [11].

Real-world examples from recent studies underscore why plants are the best raw material. Leaf extracts of Alcea rosea leaves produce highly stable, bioactive silver with superior antimicrobial, anticancer, and antioxidant activity [12]. Silver (Ag) and Gold (Au) NPs were also synthesized by using floral extracts from P. domesticum and H. sabdariffa, exhibiting good cytotoxic and antioxidant activity [13]. Nigella sativa seeds were also used for the synthesis of extremely small-sized 8-80 nm NPs [14]. Hence, NPs synthesized using the green method provide stable, biocompatible, and eco-friendly properties with enhanced antioxidant, anticancer, and antimicrobial properties [15].

However, producing nanoparticles using plant extracts faces several challenges. That includes: maintaining uniformity

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during scale-up, ensuring long-term stability and proper storage, and removing impurities from extracts are major hurdles. Due to environmentally induced variations in plant phytochemical composition, achieving consistent and reproducible nanoparticle synthesis across different batches is challenging. Also, controlling nanoparticle size and shape is tricky, as factors like pH, temperature, salt content, and reaction time can change the outcome. Overcoming these issues requires careful adjustment of reaction conditions and collaborative optimization of methods [10, 11].

To sum up, the scientific community has made joint efforts that plant-mediated synthesis represents the gold standard in green nanobiotechnology. They produce greener, safer, more cost-effective products and possess natural multifunctionality that cannot be easily copied in chemical routes. Since the world is demanding sustainable nanomaterials in biomedicine, agriculture, and environmental applications, it is high time to adopt this plant-based synthesis. Moreover, we need more investment in methods that can be scaled up, better testing in living systems, and close collaboration between researchers, industry, and regulators to tackle the remaining challenges and make the full potential of these nanoparticles a reality.

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**Review Article**

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# Biotransformation of Dairy Waste into ACE-Inhibitory Peptides: A Sustainable Strategy for Blood Pressure Management

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## ABSTRACT

The bioactive peptides produced by valorization of dairy by-products, specifically whey and casein wastes, are a sustainable approach to the treatment of environmental pollution as well as the health requirements of the population. This review is dedicated to the biotransformation of dairy waste through enzymatic hydrolysis and fermentation with the help of such strains as *Lactobacillus helveticus*, *Lactobacillus brevis*, and *Pediococcus acidilactici* to produce angiotensin-converting enzyme (ACE)-inhibitory peptides, including Val-Pro-Pro and Ile-Pro-Pro. These peptides exhibit good antihypertensive, antioxidant, and anti-inflammatory effects both in vitro and in animal models. Independent bioprocessing methods, such as enzyme immobilization and nanoencapsulation, have been used to improve the yield, stability, and bioavailability of peptides. Nevertheless, there are major shortcomings such as inconsistent effects of peptides in clinical trials, lack of human clinical trials, and the inability to scale production, yet keep costs economical. The issue of regulation is also an obstacle to translation because the approval of health claims needs to be based on strong clinical evidence and stable quality, which is not the case at present. This model focuses on waste utilization and sustainability; hence, minimizing the environmental load of dairy effluent and value addition of agro-industrial waste streams. To maximize the potential of dairy waste-based ACE-inhibitory peptides, the main agendas in the future are to put human trials first, standardize production protocols, and provide regulatory directions to attain safety, efficacy, and economic sustainability.

## INTRODUCTION

Hypertension is a major health concern in the world, and it contributes to the need to find safe and sustainable therapeutic substitutes that are also accessible. Although effective, synthetic angiotensin-converting enzyme (ACE) inhibitors have side effects and dependence. At the same time, the dairy industry produces huge amounts of protein-rich wastes, mainly of whey and casein wastes, that create major environmental and financial difficulties as a result of large biological oxygen demand (BOD), expensive waste disposal, and the loss of resources [1, 2]. Enhancing dairy waste into high-value bioactive compounds is a twofold solution: it will reduce environmental pollution and develop new nutraceuticals. Dairy proteins, particularly casein and whey, contain high concentrations of latent bioactive peptides which can be released either through enzymatic hydrolysis or microbial fermentation [3]. One of them, the ACE-inhibitor peptides like Val-Pro-Pro and Ile-Pro-Pro (fermented milk), have shown a high level of antihypertensive activity in the preclinical models [4, 5]. This review not only summarizes existing data on the biotransformation of dairy waste to ACE-inhibitory peptides, but also addresses the environmental/economic need to generate value out of dairy waste. Production strategies (fermentation, enzymatic hydrolysis) and important peptide sequences, Bioactivity, stability, and bioavailability of derived peptides, limitations, regulatory

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challenges, and future directions to clinical translation and scale-up.

**Cost-Effectiveness and Industrial Feasibility of Dairy Waste Valorization**

Cost-effectiveness and industrial feasibility of the dairy waste-derived ACE-inhibitory peptides is critical on the transition of these novel peptides into commercial products of laboratory research. The economic and practical implications of the scaling up of this valorization pathway are assessed in this section [6].

**Cost-Effectiveness: A Dual- Value Proposition**

The main economic strength is the use of cheap or negative-cost raw materials. Dairy effluents, and especially the whey, tend to pose high disposal costs to the producers because of their high biological oxygen demand (BOD) and other fees imposed on it. By re-directing this stream of waste into a production pipe-line manufacturers can be able to offset waste management costs and produce a high-value item. The price model is also good, as opposed to the de novo synthesis or isolation of peptides using high-grade food proteins [7]. The costs of processing are, however, high. Key cost drivers include. Enzyme/strain Procurement Fermentation with lactic acid bacteria (LAB) may be cost-efficient, but purified commercial proteases to perform hydrolysis are expensive. Downstream Processing steps, including ultrafiltration, peptide separation by chromatography, and encapsulation to provide stability, are energy- and capital-intensive [8]. Quality Control & Standardization: Peptide profile and potency consistency between batches can only be ensured through a strong analytical monitoring that increases the costs of operation. The balance of these production costs to the market value of the end product, which will be a high-nutraceutical or functional food ingredient, and the costs saved by not using conventional waste disposal must be presented using a full life-cycle cost analysis.

**Industrial Feasibility and Scalability Challenges**

There are a number of technical and logistical challenges in the industrial implementation. Depending on the source (e.g., cheese or casein whey) and season, dairy waste may be composed differently, which influences the consistency of the process and final peptide yield. It is necessary to standardize pre-treatment. The most practical is on-site biorefining or near-site biorefining, which is built in the current dairy processing facilities. This reduces transportation expenses of large volumes of perishable waste but involves huge capital expenditure in new bioreactors and separation facilities [9]. Although the concept of fermentation and hydrolysis at the lab scale is thoroughly documented, the transition on an industrial scale (thousands of liters) presents difficulties in sustaining the optimum pH, temperature, sterility, and mix to achieve the highest ACE-inhibitory activity. Peptides obtained using waste feeds could be subjected to increased regulatory questioning regarding safety (allergenicity, safety of possible contaminants), health claim substantiation, and then peptides obtained using food-grade feeds. Their definition as Generally Recognized as Safe (GRAS) takes a lot of paperwork.

**Comparative Feasibility with Other Valorization Routes**

Dairy waste valorization competes with other existing paths. Technological advances are technologically advanced in producing whey protein concentrate (WPC) or lactose, and may be more profit-generating on the spot. Bioactive peptides production, consequently, has to be placed in a cascading biorefinery scheme. In this scheme, waste undergoes bulk recovery (proteins as a higher-value niche, lactose), and the resulting streams are further refined into high-potency peptides to maximize resource utilization and economic gain [10].

**Conclusion on Viability**

ACE-inhibitory peptides of dairy waste can be produced industrially, but are not yet financially feasible on a large scale. It would work best in a vertically integrated dairy company with the ability to internalize waste disposal savings and use the existing infrastructure. The way forward lies in coming up with stronger and specific microbial strains or immobilized enzyme systems to enhance yield and lower the costs of processing. Separating downstream more efficiently and continuously [11]. Cultivating a clear consumer and regulatory acceptance for waste-derived bioactive compounds, supported by conclusive human clinical data. Ultimately, the driver for adoption may be as much corporate environmental, social, and governance (ESG) strategy meeting sustainability goals through circular economy innovation, as direct profit margin from the peptide products themselves.

**ACE Inhibitors**

ACE inhibitors are mostly used for hypertension control and other complications like heart failure and chronic kidney disease. This category of medicines works by preventing the activation of the angiotensin-converting enzyme (ACE) that converts angiotensin I into angiotensin II. The above-mentioned process will, in turn, cause dilation of blood vessels and a reduction in blood pressure. Talking from a historical point of view regarding the first appearance of the drug captopril in 1981 in the market, ACE inhibitors have been the principal treatment of choice, keeping high blood pressure at bay. They deliver added benefits such as improved glucose control, reduced left ventricular volumes, and cardioprotective effects, besides BP reduction [12].

**Contrasting ACE Inhibitors with ARBs**

The findings of studies are in support of the fact that both ACE inhibitors and angiotensin receptor blockers (ARBs)

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are good controls for hypertension; still, ARBs, compared to ACE inhibitors, are known to have fewer side effects, including less cough and swelling, and at the same time, they are better tolerated. This has led to ARBs being generally referred to in specific clinical settings [13]. Both drug groups seem to possess the same long-range effects on blood pressure, but ARBs' enhanced safety profile and a decline in the number of discontinuations make ARBs a better choice for some people. Nonetheless, ACE inhibitors retain their fundamental role in diabetic and heart-failure patients with fewer side effects by offering the unique means of action and additional benefits [14].

**New Findings on ACE Inhibitor Efficacy**

The latest studies bring you novel discoveries on the modalities of ACE inhibitors, which are indispensable. As an example, the combination of quinapril in the ester state with some gut bugs can facilitate the establishment of the anti-vascular wall in the technical cells system. This way, the discovery of this significant factor follows the statement of the unknown variable, which in turn can have an impact on the blood pressure, as a post-treatment reaction [15].

**Current Studies and Future Research Directions**

Scientists are now exploring new ideas for ACE inhibitors that could be specific for a single domain, multifunctional inhibitors, or peptidomimetics. Apart from ACE2, which occurred in 2000, being key in Ang(1–7) formation, the blood pressure level regulation depends on it. ACE2 also interacts with the coronavirus to become one of the standards that caused the 2003 SARS outbreak [16]. Even though hypertension is still being treated with ACE inhibitors, new facts about their mechanisms, side effects, and their relations with the microbiota change their clinical use. In contrast, ARBs are on several occasions a more suitable alternative because they have fewer adverse effects. Studies have unearthed/presented some very promising ACE-inhibitory peptides from various milk sources that may bear in the future a solution to hypertension [17].

**ACE-Inhibitory Peptides Derived from Milk Sources**

Milk and caseins underwent gastric pepsin digestion to be released in the form of peptides, for instance, PEQSLACQCL (β-lactoglobulin), QSLVYPFTGPI (β-casein), and ARHPHPHLSFM (κ-casein), which are seen as highly active ACE inhibitors. This work underlines their potential application as natural solutions to hypertension. Interestingly, casein-derived peptides, especially the α-casein ones, presented remarkable ACE-inhibiting action, and a 5-10 kDa fraction, which is the small one, brought 82.35% of the excretory activity, and the corresponding IC50 was 2.36 mg/mL. Meanwhile, β-casein-based peptides displayed ACE-inhibitory effect to a smaller extent (56.67%, IC50 4.00 mg/mL). These observations highlight the novelty of casein-derived peptides, particularly those derived from fermented dairy products, in hypertension management [18]. Moreover, a proteolytic extract derived from *Maclura pomifera* latex significantly degraded bovine caseins, and the resulting hydrolysate exhibited maximal ACE-inhibitory activity (IC50 of 1.72 ± 0.25 mg/mL), which occurred after 180 minutes. Based on the above, peptides from milk are considered advantageous in the treatment of hypertension [19]. The latest findings bring to attention the yak milk casein as a product that might lead to the development of bioactive peptides with ACE-inhibitory properties. The in-silico analysis revealed that it has a similarity to the cow milk casein, and it can produce different bioactive peptides. A study on yak milk casein from Qula, hydrolyzed by Neutrase, brought about a hydrolysate containing QC35 (IC50 0.38 mg/mL of chymase), showing its role as an antihypertensive peptide source. Calpis sour milk and related fermented milk products have been proven to have remarkable ACE-inhibitory properties. The research showed that specific peptides in fermented milk products can successfully bring down the blood pressure in hypertensive rats. This implies that there may be great benefits to treating hypertension by using fermented milk products. Amongst these, Val-Pro-Pro and Ile-Pro-Pro, peptides from the sour milk, were found to be very effective. The fermentation of *Lactobacillus helveticus* and *Saccharomyces cerevisiae* enabled the milk to be a strong ACE inhibitor through the generation of an antihypertensive peptide, which played a paramount role in vivo. These peptides were produced by Olvera-Rosales *et al.* using fermentation techniques like those applied in the current study. Yogurt, cheese, and other dairy foods also contain ACE-inhibitory peptides, which have the potential of lowering blood pressure during laboratory and animal studies; they can be used for blood pressure control [20]. A study by Wang *et al.* showed that peptides resulting from the enzymatic hydrolysis of yak milk casein possess various beneficial effects, such as ACE inhibition, antioxidant effects, anti-inflammatory action, and antidiabetic activity. Moreover, the major properties are the peptides' structure, which means they may even be food ingredients and serve as supplementary treatments for certain conditions [21]. This study aimed to investigate bioactive peptides that are found in milk in general, with a special focus on ACE inhibitors. Among these are Ile-Pro-Pro and Val-Pro-Pro, which have been recognized as ingredients that possess the capability of preventing as well as treating CVD. One of the most valuable of these peptides seems to be the one that is derived from fermented dairy products, especially cheese, despite the fact that they are often of a biodegradable and stable nature during digestion. Fermented milk products are also among those that

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provide peptides with anti-thrombotic activity, thus help where the blood clotting and platelet aggregation, hence improving the prevention of CVD. Whey proteins (alpha-lactalbumin, beta-lactoglobulin) and bovine serum albumin (BSA) also produce peptides that have ACE-inhibitory activity, some of which have added bioactivities. The most potent lactokin identified, beta-lactoglobulin f (142-148), has an ACE IC50 of 42.6.1 µmol/L, even though it is less effective than synthetic antihypertensive drugs. The ACE inhibitory effect of whey hydrolytes is, to a greater extent, a consequence of enzyme specificity than it is of the extent of hydrolysis, indicating that naturally occurring peptides could be used as functional food ingredients for the treatment of hypertension, but with drugs yielding lower potency [22]. The studies that indicated the ACE-inhibitory peptides from casein and whey proteins have been followed by. The results pointed them indirectly at the accomplishment of decreasing high blood pressure. The clinical trials done on hypertensive animals and humans who had the intention of lowering the blood pressure by dieting with these peptides, you or this society made a focused effort on functional food ingredients and nutraceuticals, thus it meant that they might reduce disease risk and aid in prevention or treatment [23]. High blood pressure, the accumulation of arterial plaque, and adult-onset diabetes are the major promoters of the risk of heart disease and stroke. The data proposes that the intake of dairy products might work as a risk factor reducing the systolic and diastolic pressure, thus protecting against hypertension and its related conditions, mainly because they contain minerals (like calcium, magnesium, potassium, and vitamin D), and they include two kinds of proteins, casein and whey protein, which can inhibit ACE [24]. The ACE-1 inhibition of angiotensin II production (a blood vessel constrictor) and the inhibition of bradykinin degradation (a blood vessel dilator) are both the regulation of blood pressure. CMPs obtained from cow, sheep, and goat milk products also display moderate ACE-inhibitory activity, but that effect intensifies by the process of the simulated digestion mechanism present in the stomach and intestines [24]. The activity of the CMPs as inhibitors of the ACEs is significantly increased upon trypsin-induced degradation, thereby giving rise to the fragments that have a stronger ACE-inhibitory activity than the original CMPs. This suggests that it could be possible to make cheese whey work more efficiently in the making of health-promoting products and supplements with an ACE-inhibitory effect. Milk caseins and whey proteins, which are the most abundant sources of the bioactive peptides that have various health benefits such as opioid-like activity, immune-enhancing, ACE-inhibitory, lowering blood pressure, and antimicrobial effects, have been identified as such. Among them, the peptides that inhibit the activity of ACE and lower the blood pressure are extensively researched because their manner of action might be useful in the treatment of hypertension. Milk has been thought of as the best source of the derivative of these types of beneficial peptides compared to other alternatives, thus it is a key material in the functional food industry. When goat milk proteins were cleaved by subtilisin and trypsin enzymes, the resultant product mixture exhibited strong ACE inhibitory activity (IC50 of 218.50 µg/mL) [25]. An additional process through a 50 kDa ceramic filter increased the activity of more than 30%, suggesting potential for optimization of the food products that aim at blood pressure regulation. Whey protein impurities, as a by-product, have been shown to have ACE inhibitory action in different experimental conditions. A certain product formed from β-lactoglobulin given IC50 of 28 µg/mL; it also contained new and specific peptides such as Ile-Ile-Ala-Glu (IIAE). This suggests its power of lowering blood pressure. The peptide Tyr-Pro, present in alpha s1-casein, beta-casein, and kappa-casein, seemed to have a powerful hypotensive action on spontaneously hypertensive rats. These actions were time-dependent; they started 6 hours after ingestion [26]. Although Tyr-Pro has a weaker activity against ACE (IC50 of 720 µM), its mechanism of lowering blood pressure appears different and can change old theories about blood pressure control [23]. The studies tested the immunopositive domino effect of the monoclonal antihuman sMSTN impurity in becoming the main antibody response in the ELISA system for the detection of the peptides. The developed ELISA could specifically detect the increasing T-cells and the cytokine TNF-α, while TGF-β1 and the vasodilatory prostaglandin E1 increased and activated MAC-1 on sow no. 4 to inhibit the virus [27]. The reaction was carried out by the MMZ-F-017 bacto-screen method, which concentrated the sample on a nitrocellulose membrane fixed to a plasmid [13]. The chromatographic separation of the three components, namely α(S1)-casein, β-casein, and α(S2)-casein, was carried out in the present study, and the presence of three bioactive peptides exhibiting ACE inhibitory, immunomodulatory, and antimicrobial properties was confirmed in fresh milk proteins [28]. These discoveries point to the wide potential of the milk proteins with respect to diverse health effects. In like manner, ultrafiltration characterizing protein fragmentation and the enzymatic interaction of caseins with the enzyme in the production of hydrolysates with astringent ACE inhibition action demonstrates the therapeutic nature of milk for hypertension treatment [16]. A critical gap in translating milk-derived ACE-inhibitory peptides into therapeutic recommendations is the lack of clear dose equivalence with pharmaceutical ACE inhibitors (e.g., captopril, enalapril) [29]. Synthetic drugs are developed with high

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specificity and potency, typically exhibiting in vitro $IC_{50}$ values in the nanomolar range. Conversely, the most potent milk peptides, including Val-Pro-Pro, have IC 50 values in the micromolar scale, meaning that they are orders of magnitude weaker on a molar basis. Thus, to obtain a similar antihypertensive effect, gram quantities of peptides would have to be used, which is in comparison with milligram doses of synthetic drugs. Such a difference is further compounded by deep variations in bioavailability and presystemic degradation. Pharmaceutical ACE inhibitors are chemically designed so that they are most effectively absorbed and metabolically stable, and in many cases, they have a bioavailability that is greater than 60%. Milk peptides, in turn, are prone to extensive degradation by gastrointestinal proteins and have low membrane permeability, resulting in low (<1-5) and very variable systemic absorption [30]. This means that the effective dose that is carried to target tissues/circulatory system is a minute fraction of the dose ingested. To conclude, the intrinsic ACE-inhibitory activity of milk peptides, as well as the deliverable systemic concentration are significantly lower than that of synthetic drugs. This does not rule out their possible usefulness as functional food ingredients for the mild support of blood pressure, but it clearly classifies them as opposed to pharmaceutical-grade interventions. They are to be considered as complementary agents to be used in a lifestyle management program, as opposed to their direct, milligram-to-milligram replacement of first-line antihypertensive drugs.

**Variables Impacting ACE Inhibition and Cell Viability:**

Various factors, such as fermentation temperature, whey powder, calcium lactate, soybean peptone, glucose, and casein, have a direct impact on the ACE inhibition and viable cell count in the fermentation process of *Lactobacillus bulgaricus* LB6. High temperatures, whey powder, glucose, and calcium lactate are responsible for stronger inhibition of ACE, whereas soybean peptone and casein seem to have the opposite effect [31].

**Improving Fermentation Conditions**

A Box-Behnken design of response surface methodology was employed to yield maximum ACE-inhibitory peptide production from goat milk using *Lactobacillus bulgaricus* LB6 [32]. The best results with a total viable count of 8.06 × 10^7 and ACE inhibition rate of 86.37% were achieved when 0.15% casein, 1.2% glucose, and 0.35% soybean peptone were used. Furthermore, a second study concluded that *Lactobacillus bulgaricus* LB6 improved the most when a 12-hour fermentation time was used, and the following components: whey powder (0.70%), casein peptone (0.90%), soybean peptone (0.30%), and casein (0.20%) all helped the inhibitions ACE [33].

**Specific Strains and their ACE-Inhibitory Efficacy**

The fermentation products of *Lactobacillus animalis* DPC6134 contained ACE inhibitory compounds that showed an activity of 85.51% and an IC50 value of 0.8 mg/mL. Simultaneous growth of *Lacticaseibacillus rhamnosus* GG and *Streptococcus thermophilus* SY-102, which extended the exponential phase from 12 to 24 hours, facilitated the breakdown of proteins and increased ACE inhibition (53.42%) when compared to the one-strain fermentation [34]. Lactobacillus brevis, Lactobacillus helveticus, and *Lactobacillus paracasei* were the most successful in converting whey protein to ACE-inhibitory peptides, and they reached the inhibition rates between 93.3% and 100% [35].

**Novel Techniques for Generating Ace-Inhibitory Peptides: Enzyme Immobilization**

Optimizing *Lactobacillus helveticus* LB10 proteinases by attachment to a surface led to an increase in whey protein degradation and thus improved their sustainability and reusability [36].

**Nanoencapsulation**

Injecting Aspergillus oryzae fungal protease into nanoparticles properly released ACE-inhibitory peptides during casein degradation [37].

**Identification and Examination of Bioactive Peptides**

Karthikeyan *et al.* have identified whey protein hydrolysates that contain several biologically active peptides, such as those resistant to the action of DPP-IV, others being of antibacterial nature, and having the ability to block ACE [38]. Whey-based caseinate hydrolysates from different whey sources contained ACE-inhibitory peptides with very low IC50 values (16 to 100 µg/mL) [39].

**Stability and Uptake of ACE-Inhibitory Peptides: Gastrointestinal Resilience**

ACE-inhibitory peptides from milk fermented by *Lactobacillus helveticus* KLDS.31 and *Lactobacillus casei* KLDS.105 remained bioactive after simulated digestion [40].

**Temperature and pH Stability**

The Gly-Ala (GA) dipeptide that is produced by *Lactobacillus plantarum* QS670 fermented whey protein with a stable behavior to a wide variety of temperatures, pH levels, and digestive enzymes was reported in their study [41].

**Peptide-Specific Stability**

The beta-casein peptides showed resistance to gastric enzymes, and they are readily broken down by pepsin [42].

**Animal Trials and Antihypertensive Effects**

*Lactobacillus helveticus* strains R211 and R389 used casein during fermentation, which caused lowering of the blood pressure in notable degrees in the hypertensive rats used [43].

***Pediococcus acidilactici* SDL1414 for ACE-Inhibitory Peptide Production**

The Pealo +4 containing lactic acid bacteria were 34, and *Pediococcus acidilactici* SDL1414 was the most potent

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(84.7%, IC50 = 19.78 µg/mL)[44]. Furthermore, 57.7% of the low molecular weight peptides (<7 kDa) had been discovered to block ACE. Hence, they were termed as ACE inhibitors as well[45].

**Limitations and Future Perspective**

Although substantial evidence supports the ACE-inhibitory potential of dairy waste-derived peptides, most findings are based on in vitro and animal studies, with limited confirmation from human clinical trials. Variability in fermentation conditions, peptide composition, and bioavailability further constrains standardization and clinical translation. Future research should therefore focus on well-designed human trials, harmonized production protocols, and advanced delivery strategies to enhance efficacy, safety, and regulatory acceptance.

## CONCLUSION

The ability to convert dairy waste into ACE-inhibitory peptides is an attractive dual-purpose approach that offers the solution of managing sustainable waste, as well as the need to design potential hypertension nutraceuticals. The preclinical studies have a solid basis on the in vitro and animal-model efficacy of the peptides like Val-Pro-Pro and Ile-Pro-Pro. Nevertheless, there is a critical translational gap between converting this promise into commercially viable products that have been translated into clinically valid products. Great barriers still exist, such as the cost and complexity of definitive human trials, unresolved issues with standardizing the potency and with consistent bioavailability and the demanding regulatory environment that requires strong safety and efficacy information, especially of substances obtained via waste streams. Advanced bioprocessing methods can provide the route to the better yield and stability, but the final success of the new approach relies not only on the technical feasibility but on the ability to demonstrate the clear cost-effectiveness, regulatory approvals, and therapeutic value in the long-term human trials. Future directions should then shift toward descriptive research to focused translational and clinical studies which directly touch upon these economic, regulatory and scientific obstacles to achieve the sustainable potential of dairy waste-derived bioactive peptide.

## Authors' Contribution

Conceptualization: RY
Methodology: RY
Formal analysis: AS, AA, HA
Writing and Drafting: AS, AA, HA
Review and Editing: RY, AS, AA, HA

All authors approved the final manuscript and take responsibility for the integrity of the work.

## Conflicts of Interest

All the authors declare no conflict of interest.

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**Review Article**

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# CRISPR-Mediated Engineering of Lignin Biosynthesis to Reduce Plant Biomass Recalcitrance: Advances, Trade-offs, and Future Directions

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## ABSTRACT

Growing demands on fossil fuels and population growth have increased the need for sustainable and renewable energy sources on a worldwide scale. Lignocellulosic biomass can be used as a feedstock to make biofuels. However, a variety of challenges, such as low yields and expensive treatment costs, prevent biomass commercialization due to its recalcitrant nature. One of the primary sources of this resistance is lignin, a substantial component of the cell wall. The ability to precisely alter the genes involved in lignin formation has been made possible by recent advancements in CRISPR/Cas-based genome editing, opening up new possibilities to improve biomass quality without sacrificing plant growth. This paper discusses current developments in CRISPR-based lignin engineering, targetable lignin biosynthesis genes, and associated agronomic and phenotypic results. Furthermore, it highlights critical challenges, including the need for precise regulation, integration of multi-omics techniques, long-term field evaluation, and balancing biomass processability with plant health for sustainable bioenergy production.

## INTRODUCTION

Energy is one of the driving forces behind economic expansion. However, the extensive use of fossil fuels led to several negative environmental issues, including global warming, air pollution, and depletion of non-renewable resources [1]. These challenges have accelerated the demand for renewable and sustainable energy sources, with biofuels gaining attention. Biofuels are produced from organic materials such as lignocellulosic crops, agricultural leftovers, and other biomass sources. Microbial fermentation is typically used to convert biomass into fermentable sugars [2]. Second-generation biofuels, derived from lignocellulosic biomass, corn, wheat straw, and sugarcane residues, offer a promising alternative to fossil fuels [3]. Lignocellulosic biomass is composed of three major components, cellulose (40-60 wt%), hemicellulose (20-40 wt%), and lignin (10-25 wt%), and represents an abundant and renewable resource for biofuel production [4]. However, biofuel production from lignocellulosic biomass is hindered by the recalcitrant nature of lignin [5]. Lignin restricts biomass digestibility by reducing cell wall accessibility and inhibiting enzymatic hydrolysis [6]. Genetic manipulation of lignin production has emerged as a promising tool for enhancing biomass conversion efficiency. Enzymatic digestibility can be improved by downregulating lignin-related genes, which can lead to unfavourable impacts on plant growth and

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development [7]. Recent breakthroughs in genome editing technologies, particularly CRISPR/Cas9, have made it possible to target lignin production genes precisely to change lignin content and composition in a variety of crops, including barley, switchgrass [8, 9].

Here, several research gaps are filled to assist in striking a balance between biomass quality and plant health, enabling the more economical and efficient generation of sustainable biofuel from lignocellulosic biomass. This study aims to investigate the agronomic and phenotypic effects of lignin alteration and outlines important lignin biosynthesis genes that CRISPR/Cas systems target. It also discusses biosafety laws about altered plants, research gaps, and present difficulties.

**Lignin Biosynthesis and Its Role in Biomass Recalcitrance**

Cellulose, hemicellulose, and lignin are the three main constituents of lignocellulosic biomass [10]. In the lignin and hemicellulose matrix, cellulose creates beta 1-4 glycosidic bonds between glucose molecules to produce microfibrils [11]. Plants are given a strong structural framework by lignin, a heteropolymer comprising p-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) monolignol that encircles cellulose microfibrils in a complicated branching network [5]. As a plant ages, its lignin content rises. Only a few S units are seen in the early phases of lignin deposition, whereas the integration of H and G units begins at lignification. Coniferyl alcohol and sinapyl alcohol are then added to create a mixture of G and S units during secondary wall development [12]. The lignin mass percentage ranges from 9 to 30% for the majority of woody feedstock species, while it is believed to be lower for Agave, ranging from 5 to 16% depending on the species [13]. Lignin is resistant to hydrolysis; therefore, lowering the bulk fraction of lignin or changing its structure are crucial objectives for overcoming the recalcitrance and enhancing saccharification.

**Target Genes in the Lignin Biosynthetic Pathway**

The phenylpropanoid pathway is a complicated process that involves many enzymes for monolignol production and lignin biosynthesis. These enzymes can be targeted to reduce the lignin content in plants. The CRISPR-Cas tool can be used to mutate the genes that are involved in their synthesis.

**Upstream Genes (PAL, C4H, 4CL)**

Phenylalanine ammonia-lyase (PAL) catalyses the first step of the phenylpropanoid pathway by changing phenylalanine into trans-cinnamic acid. It is coded by multiple genes having different isoforms, abundant in wood-forming tissues, and localized in the endoplasmic reticulum [14]. Cinnamate 4-hydroxylase (C4H) is a cytochrome P450 monooxygenase of the CYP73A family. It catalyses the hydroxylation of cinnamic acid to form p-coumaric acid in the lignin synthesis pathway. It has two primary isoforms: Class I is involved in lignin production and expressed in the endoplasmic reticulum; Class II is involved in stress responses and has tissue-specific expression [15]. 4-Hydroxycinnamoyl-CoA (4CL) initiates the synthesis of hydroxycinnamoyl-CoA esters. In Eucalyptus, many 4CL genes form clusters and are involved in lignin synthesis with class I isoforms and localized in the endoplasmic reticulum. Several phenylpropanoid-derived pathways involve class II and 4CL-like proteins [16].

**Midstream Genes (HCT, C3H)**

Shikimate hydroxycinnamoyl transferase (HCT) converts hydroxycinnamoyl groups into shikimate or quinate, creating the intermediates needed for additional hydroxylation processes. Several HCT gene clusters, with distinct isoforms, are expressed in specific lignifying tissues [17]. *p-Coumarate* 3-hydroxylase (C3H) is a CYP98A cytochrome P450 enzyme that hydroxylates p-coumaroyl shikimate or quinate esters to form caffeoyl derivatives. Many C3H isoforms are known to exist and are expressed in the ER. Pathway regulation and lignin composition may be influenced by functional heterogeneity among isoforms [18].

**Downstream Genes (CCoAOMT, CCR, F5H, COMT, CAD)**

Caffeoyl-CoA O-methyltransferase (CCoAOMT) is a crucial precursor of lignin monomers. Feruloyl-CoA is created when CCoAOMT catalyses the methylation of caffeoyl-CoA. Two main CCoAOMT isoforms are found in the cytoplasm and are significantly expressed in lignifying tissues [19]. Cinnamoyl-CoA reductase (CCR) catalyses the reduction of hydroxycinnamoyl-CoA esters to aldehydes. Only some isoforms are functionally linked to lignin production [20]. Ferulate 5-hydroxylase (F5H) is a cytochrome P450 enzyme that belongs to the CYP84 family; it aids in the production of syringyl lignin by facilitating the hydroxylation of ferulate derivatives. The most highly expressed F5H isoforms are found in the ER and play a key role in lignin production [21]. Caffeic acid O-Methyltransferase (COMT) catalyses methylation processes, converting hydroxylated intermediates into precursors of sinapyl alcohol. COMT is one of the most prominent lignin biosynthetic enzymes in Eucalyptus, indicating that it plays an important role in lignin production. There are several COMT-like genes, but only a few are important for lignification [22]. Cinnamyl Alcohol Dehydrogenase (CAD) catalyses the last stage of monolignol biosynthesis, which transforms cinnamyl aldehydes into their corresponding alcohols. Only some part of the many CAD isoforms is closely linked to the formation of lignin [23]. In addition to biosynthesis, chitinases, laccases, and dirigent proteins influence lignin polymerization and structure [24]. Laccases are essential enzymes that catalyse the oxidative polymerisation of monolignols into lignin without the need for $H_2O_2$ [25]. Dirigent-like proteins are involved in regulating the

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structure of lignin and aid in the stereoselective binding of monolignols [26]. Although their functional roles are still unclear, these targets provide an alternate approach by altering the architecture of lignin rather than its quantity. The Phenylpropanoid pathway of lignin biosynthesis was analyzed [10] (Figure 1).

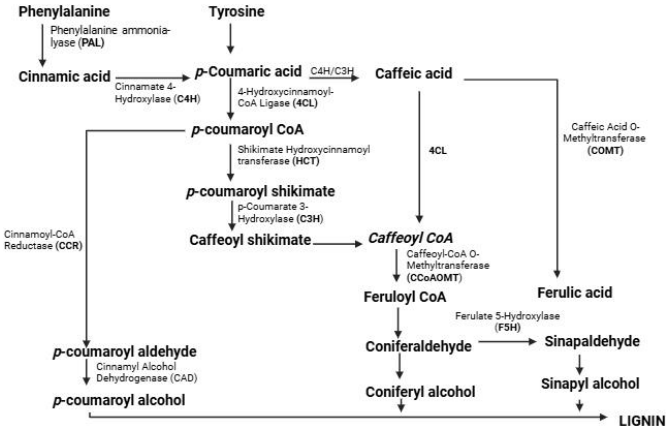


**Figure 1:** The Phenylpropanoid Pathway of Lignin Biosynthesis

## Comparative Analysis of Target Genes

PAL, C4H, 4CL control carbon flux into the pathway. Although editing these genes typically results in significant lignin reduction, the disruption of vital metabolic processes frequently causes severe growth abnormalities [15, 16]. The production and distribution of lignin precursors is regulated by midstream genes (HCT, C3H). Studies have shown that mutations in these enzymes can significantly reduce the concentration of lignin, but the outcomes have been inconsistent, ranging from enhanced saccharification to severe dwarfism. This variation is a reflection of route flexibility and metabolic compensation [17, 18]. More intriguing targets are downstream genes (CCoAOMT, CCR, F5H, COMT, and CAD). Editing these genes frequently modifies the lignin's composition rather than its total concentration, particularly the S/G ratio, which has an impact on biomass digestion. For example, F5H and COMT alteration increases syringyl lignin, enhancing processing efficiency without a large growth penalty [21, 22]. CAD downregulation, which increases digestibility without appreciably reducing lignin concentration, further highlights the importance of compositional engineering over bulk reduction [23]. No single gene target provides an optimal solution. Upstream targets maximize lignin reduction, whereas downstream targets offer a better balance between biomass quality and plant performance. Therefore, combinatorial and fine-tuned editing strategies are more effective than single-gene knockouts.

## CRISPR Technologies Applied to the Lignin Pathway

CRISPR/Cas9 is a ground-breaking genome editing tool to modify the genome by introducing double breaks in the DNA, directed by sgRNA. The Cas9 enzyme is responsible for locating and attaching itself to target DNA sequences. After binding, Cas9 marks the site of DNA cleavage by generating a protospacer adjacent motif (PAM). The combination of two RNA molecules, trans-activating crRNA (tracrRNA) and crRNA, known as single-guide RNA (sgRNA), facilitates the assembly of Cas9 in this process [27]. A single DNA construct knocks out the expression of an endogenous gene involved in lignin monomer biosynthesis while simultaneously expressing an altered version of the gene's open reading frame that is not cut by the Cas9 system, complementing the introduced mutation in the lignin biosynthesis pathway. CRISPR/Cas9 has several advantages over traditional genetic engineering. Its precision allows specific targeting and reduces unintentional off-target effects and genetic manipulations; efficiency produces first-generation homozygous mutants [28]. It targets multiple genes at a time and modifies complex traits with minimal growth penalty in lignin-reduced mutants [29]. Research has been done on different plants like barley, switchgrass, poplar, etc., to reduce their lignin content using CRISPR/Cas9.

## Quantitative Comparison of Lignin Engineered Plants

Multiplex CRISPR editing in poplar achieved significant lignin reduction (up to 51.7%), surpassing single-gene edits like CSE (~29%). While barley COMT1 editing resulted in 14% lignin decrease but a 34% increase in glucose recovery, 4CL1 mutants in switchgrass demonstrated up to 30% lignin reduction with the biggest improvements in sugar release. Multi-gene targeting (e.g., CCR and CAD) in poplar reduced lignin by 20–40% and increased ethanol yield by 30–50%, outperforming single-gene approaches, although it required extensive haploid screening [30, 31]. Similarly, compared to woody species, maize PAL knockout resulted in a 15–25% decrease in lignin and a ~40% increase in glucose release, along with faster regeneration. Studies showed defense-related lignification is improved by regulatory gene editing (e.g., WRKY16), but growth is severely limited. CRISPR-based strategies show improved efficiency with lower penalties (~0–5%) and 1.2–4-fold increases in saccharification when compared to traditional RNAi approaches, which reduce lignin by ~20–30% but suffer 10–20% growth penalties [32-34]. These results demonstrate that while increased lignin reduction is associated with increased biofuel yield, the best results require striking a balance between biomass digestibility, plant growth, and structural integrity.

## Research Trends in Global Lignin Engineering

Global trends highlight CRISPR for bioenergy crops, with market growth from 415 M (2025) to 600 M (2034), with Cas9 (65% share) shifting to base/prime editors (8.2% CAGR). The US focuses on therapeutic crossover, Europe on poplar for forestry, and Asia-Pacific on plant applications (such as barley and switchgrass). AI-optimized sgRNAs, RNP delivery for perennials, and multiplex edits aimed at

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phenylpropanoid redundancy, projected 22% CAGR to $6.92 B by 2030, are among the trends. Low-lignin grasses are given priority in CRISPR trials that target biofuels [35] (Table 1).

**Table 1:** Summary of CRISPR/Cas-Engineering for Lignin Reduction in Plants

| Study | Plant Species | Key Outcomes | Lignin Reduction | Saccharification/Glucose Release Improvement | Growth Penalty | References |
|---|---|---|---|---|---|---|
| CSE Knockouts | Hybrid Poplar | Mutants in a single or both genes; no morphological changes | 29.1% | +25% | None | [30] |
| PDS Activation | Poplar | 51.7% efficiency; 30/59 homozygous mutants | N/A (control study) | N/A | Not reported | [28] |
| HvCOMT1 Knockout | Barley | Improved biofuel yield; no morphological changes | 14% | Glucose recovery: +34%; Bioethanol:14.3 g/L (+34% vs. WT); Sugar yield:0.46 g/g (+12% vs. WT) | None | [31] |
| CCR1 Knockout + Complementation | Poplar/ Arabidopsis | T1 vessel-specific rescue; T2 homozygous individuals show a strong negative phenotype without construct | Variable (Strong allele control) | 4x sugar/plant vs. 2x mutants | Strong growth penalty without complementation (dwarfism-like); rescued to normal | [32] |
| WRKY16 Knockout | Tomato/ Arabidopsis (vs. *parasitic plants*) | Constant lignin accumulation for defense | Increased (sustained) | N/A (defense focus) | Compromised vegetative growth (trade-off) | [33] |
| RNAi Lignin | Sugarcane | Field-tested suppression | 20–30% | Improved | Dwarfism; lodging; pest vulnerability | [34] |

**Advancement in Engineering Tools**

In addition to CRISPR/Cas9, which uses sgRNA-guided Cas9 and PAM recognition to create double-strand breaks, more recent versions address precision constraints for lignin engineering. Base editors, such as adenine base editors (ABE) and cytosine base editors (CBE), allow single-nucleotide changes without breaks, changing C-G to T-A or A-T to G-C, covering about 60% of disease variants and lowering off-target risks, which is perfect for fine-tuning lignin genes like 4CL or COMT without indels. With recent proPE variants increasing efficiency 6.2-fold and accuracy to 1/101 error rate, prime editing installs any of 12 base changes, small insertions/deletions, and is appropriate for precise syringyl unit modifications in polyploid crops. While RNP complexes and lipid nanoparticles enhance delivery in resistant plants, CRISPR/Cas12a (Cpf1) provides alternative PAM sites and staggered cuts for multiplexing. These hold promise for lignin pathways, though plant applications lag behind human therapeutics [36].

**Biological and Technical Limitations of CRISPR-Mediated Lignin Engineering**

CRISPR/Cas has biological and technological limits despite its accuracy and effectiveness. Optimizing genome editing techniques and converting CRISPR-mediated lignin engineering into agronomically viable crops requires an understanding of these limitations.

**Gene Redundancy and Metabolic Compensation**

Gene redundancy and a plant's capacity to adjust physiologically after gene disruption are two of the most important biological difficulties. Numerous enzymes are involved in the phenylpropanoid pathway, which produces lignin. Alternative gene isoforms can restore the function of a knocked-out gene in a single gene knockout. Functional redundancy was shown in a study on hybrid poplar, where some mutant lines showed considerable lignin reduction while others showed modest phenotypic effects despite gene editing. The predictability of lignin engineering is limited by the densely linked metabolic networks of plants, which have the potential to reprogram biosynthesis [37].

**Off-Target Effects in Complex Plant Genomes**

Off-target mutations in large polyploid genomes by CRISPR are still a significant concern. Even though the CRISPR system is very precise, imperfect base pairing guided by sgRNA can cause unintended cleavage at homologous genomic sites. CRISPR/Cas systems can introduce unintended mutations at off-target sites, affecting gene function [38].

**Mosaicism and Chimerism in Regenerated Plants**

Mosaic plants contain a mixture of edited and unedited cells, which results in an incomplete phenotype, because CRISPR editing continues after the initial transformation event, leading to independent editing events in different cell lineages during plant development, and results in plants with fully edited cells, partially edited cells, and Unedited cells. This leads to unequal lignin distribution affecting vascular function and mechanical strength [39].

**Species-Specific Challenges in Recalcitrant Crops**

CRISPR-mediated lignin engineering faces species-specific barriers, especially in woody and recalcitrant crops. Polypoid species have multiple homologous copies of lignin biosynthesis genes, required to edit multiple

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alleles to get effective results. Precise spatial and temporal editing is required to prevent growth abnormalities because lignin deposition is strictly controlled during vascular differentiation [40]. Addressing these limitations is essential for the successful translation of CRISPR-mediated lignin engineering into sustainable bioenergy crops, improved forage species, and industrial biomass feedstocks.

**Phenotypic and Agronomic Consequences of CRISPR-Based Lignin Modification**

CRISPR/Cas editing reduces total lignin content and changes monomer ratios to increase cell wall porosity and digestibility. CRISPR/Cas tool targeting multiple genes produced mutant poplar plants with 50% reduced lignin content and up to ~28% increase in carbohydrate-to-lignin ratios, enhancing enzyme accessibility [31]. Change in lignin content, composition, or deposition can cause morphological changes in plants. Classic lignin modification leads to dwarfed stature, collapsed xylem, impaired water transport, abnormal leaf structure, or reproductive abnormalities, due to disruption in lignin's structural role [41]. CRISPR/Cas was used to mutate the SoLIM transcription factor in sugarcane. The mutant lines showed 9.7%–51.5% reduction in total lignin content, altered S/G monomer ratio, histochemical and microscopy analysis confirmed a thinner cell wall and a normal phenotype. Lower lignin sugarcane could reduce costly pretreatments in biofuel production and improve process economics, without obvious productivity loss [42].

**Growth-Defense Trade-Offs**

In plants, limited metabolic resources like carbon, nitrogen, and energy cause growth-defense trade-offs to maintain fitness, protective metabolism, and biomass accumulation [43]. The defence-growth barrier is compromised when lignin production is disrupted, leading to energetically induced defence responses that divert resources from growth. By returning defence gene expression and resource allocation to normal, suppressor mutations, including those affecting Mediator components, can partially restore growth. Although suppressor mutations can temporarily restore growth and defense gene expression, lignin-deficient plants frequently exhibit decreased stature due to the ongoing metabolic expenditures of induced defense responses [44]. As metabolic fluxes are complicated, quantitative knowledge of growth-defense trade-offs in lignin-modified plants is limited. Quantitative proteomics and stable isotope labelling in conjunction with LC-MS can help to clarify resource dynamics. It will be crucial to comprehend and adjust these trade-offs if lignin-engineered plants are to be successfully implemented in their natural environments [45]. It is possible to anticipate the best resource allocation for lignin-engineered crops under natural environmental settings by incorporating these observations into constraint-based metabolic models [46].

**Long-Term Field Trials**

The majority of research on crops modified with lignin is carried out in greenhouses or controlled environments, which do not accurately reflect environmental variability. To assess agronomic stability, stress tolerance, pathogen susceptibility, and biomass yield across seasons, long-term field studies are crucial. In the field evaluation of lignin-engineered poplar, it was observed that, despite the low lignin enhancing saccharification, various lines were more susceptible to environmental stress and exhibited altered growth performance over the years. These findings demonstrate that the enhancement in the lab digestibility might not necessarily translate into uniform field performance, and, thus, the need to conduct multi-location and multi-year assessments [47].

**Limitations and Future Perspective**

Despite tremendous progress, there are still a number of important research gaps, including growth penalties, vascular issues, and decreased stress tolerance. Combining multi-omics techniques is necessary to properly understand growth-defence trade-offs and metabolic compensatory mechanisms. Future research should prioritize precision editing techniques that allow for fine-tuning of lignin concentration and composition over severe reductions. The plants' resistance, which currently have lower lignin content (for example, agave has a lignin content of 5–16%), should be the focus of research. Another important gap is the insufficient field-based and long-term evaluation of lignin-engineered crops. Regulations governing genome-edited crops, including CRISPR-modified lignin variants, are always evolving on a global scale. Significant biosafety issues include off-target mutations, gene flow to wild relatives, and unforeseen ecological effects such as altered plant-microbe or plant-insect interactions. In terms of ecology, lignin loss may impair plants' natural defences and structural integrity, leaving them more susceptible to pests and illnesses that might disrupt ecosystem dynamics. Agronomic, ecological, and molecular data must be included in risk estimation frameworks before commercialization.

## CONCLUSION

Lignocellulosic biomass is a significant renewable resource for biofuel production; however, lignin-induced recalcitrance is a significant limitation. CRISPR/Cas genome editing reduces lignin biosynthesis/accumulation to improve biomass processing across multiple crops, including poplar, sugarcane, Arabidopsis, and barley. Studies showed successful results with some challenges, including growth-defense trade-off and morphological

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penalties like dwarfism. Additionally, Large-scale commercialization is hindered by high production costs and a lack of field validation. Therefore, Precision editing techniques, improved knowledge of transcriptional and metabolic control, optimization of biomass quality, and plant fitness will be necessary for future advancements. Achieving this balance is necessary for the effective execution of lignin engineering for bioenergy applications.

## Authors' Contribution

Conceptualization: HA
Methodology: HA
Formal analysis: HA
Writing and Drafting: HA
Review and Editing: HA

All authors approved the final manuscript and take responsibility for the integrity of the work.

## Conflicts of Interest

All the authors declare no conflict of interest.

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**Original Article**

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# High ColabFold Confidence Does Not Guarantee Catalytic-Site Accuracy in *Bacillus subtilis* PdxT

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## ABSTRACT

AI-based protein structure predictors such as AlphaFold2 and ColabFold routinely generate models with high confidence scores for backbone geometry. However, whether these global metrics reliably capture catalytically competent active-site configurations in enzymes remains unclear. **Objective:** To evaluate whether a high-confidence ColabFold model of the glutaminase subunit PdxT from *Bacillus subtilis* accurately reproduces the geometry of its catalytic cysteine–histidine–glutamate triad. **Methods:** The amino acid sequence of *B. subtilis* PdxT (UniProt P37528) was submitted to ColabFold v1.5. Five models were generated with default settings, AMBER relaxation, and MMseqs2-based multiple sequence alignment. The top-ranked model was selected based on predicted Local Distance Difference Test (pLDDT) and predicted TM-score (pTM). Inter-residue distances between Cys118, His168, and Glu51 were measured from the predicted structure and compared with distance ranges (2.5–5.0 Å) reported for experimentally solved Class I glutaminase structures. **Results:** The top ColabFold model displayed high global confidence (mean pLDDT 96.4; pTM 0.929). The measured inter-residue distances were 10.36 Å (Cys118–His168) and 18.0 Å (His168–Glu51), exceeding the 2.5–5.0 Å range typically required for catalytic function. No experimental validation or additional computational analyses were performed. **Conclusions:** In this PdxT model, high global confidence metrics did not correspond to catalytically realistic active-site geometry. These findings suggest that AI-generated protein models intended for functional interpretation may require secondary validation focused on active-site architecture.

## INTRODUCTION

Vitamin B6, which includes pyridoxine, pyridoxamine, and the active cofactor pyridoxal 5'-phosphate (PLP), is an essential molecule involved in numerous biological processes, including amino acid metabolism, neurotransmitter biosynthesis, one-carbon metabolism, heme formation, and immune regulation [1]. Although PLP is universally required across living systems, organisms differ substantially in their ability to synthesize it de novo. Bacteria, fungi, plants, and archaea retain complete vitamin B6 biosynthetic pathways, whereas humans and most animals lack these genes and therefore depend on dietary intake to meet metabolic demands [2]. In bacteria, the terminal step of PLP biosynthesis is catalyzed by the PdxS/PdxT enzyme complex [3]. PdxT functions as the glutaminase subunit, hydrolyzing glutamine to release ammonia, which is subsequently transferred through an inter-subunit ammonia tunnel to the PdxS synthase subunit [4]. This tightly coupled architecture prevents loss of the reactive intermediate and enhances catalytic efficiency [5]. Beyond its metabolic importance, the PdxS/PdxT system represents an attractive antimicrobial drug target. The pathway is absent in humans but is conserved in numerous pathogenic bacteria, including *Bacillus anthracis*, *Streptococcus pneumoniae*,

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*Mycobacterium tuberculosis*, and *Salmonella* species [6]. Genetic studies further indicate that pdxT is essential for bacterial survival under vitamin B6-limiting conditions, supporting its potential as a selective therapeutic target [7]. Despite this significance, structural characterization of PdxT remains limited, and most available computational studies rely on homology models that provide only partial insight into active-site architecture [8]. Recent advances in artificial intelligence-based protein structure prediction have transformed structural biology. AlphaFold2 and its optimized implementation ColabFold enable rapid, high-accuracy prediction of protein folds directly from sequence information [9, 10]. These tools routinely produce models with high global confidence metrics, such as predicted Local Distance Difference Test (pLDDT) scores and predicted TM-scores (pTM), which are widely interpreted as indicators of structural reliability. However, these metrics primarily reflect confidence in backbone topology and overall fold. This study hypothesized that high global confidence metrics (pLDDT >90, pTM >0.9) would correspond to accurate active-site architecture with inter-residue distances within the 2.5–5.0 Å range characteristic of catalytically competent Class I glutaminases. Testing this hypothesis is essential for determining the suitability of AI-generated enzyme models for functional inference and structure-based drug design.

Critically, whether such global confidence measures reliably reflect catalytically competent active-site geometry—especially in enzyme systems requiring precise spatial positioning of catalytic residues—remains insufficiently examined. This gap is particularly relevant for PdxT, whose glutaminase activity depends on accurate spatial organization of a conserved Cys–His–Glu catalytic triad. Most validation studies have focused on global fold accuracy rather than functional site precision, creating uncertainty about the suitability of AI-generated models for mechanistic interpretation or drug discovery applications. Therefore, this study aimed to evaluate whether a high-confidence ColabFold model of *Bacillus subtilis* PdxT accurately reproduces the catalytically relevant geometry of its Cys–His–Glu triad.

## METHODS

This descriptive computational structural analysis evaluated whether high global confidence metrics generated by AI-based protein structure prediction correspond to catalytically competent active-site geometry. The analysis was limited to structural assessment; no experimental validation, molecular docking, molecular dynamics simulations, or inferential statistical testing were performed [11]. The amino acid sequence of *Bacillus subtilis* PdxT (UniProt accession: P37528; 196 amino acids) was retrieved in FASTA format from the UniProt database (https://www.uniprot.org/). The native sequence (molecular weight ~21.8 kDa; theoretical pI 5.47) was used without truncation or modification to ensure methodological reproducibility. Protein structure prediction was performed using ColabFold v1.5, an accelerated implementation of AlphaFold2 optimized for GPU-based computation. Predictions were executed using a Google Colab GPU runtime (NVIDIA Tesla T4) with the following default parameters: Multiple sequence alignment (MSA): MMseqs2-based search against UniRef30 and environmental sequence databases with three iterations, Template search: Enabled (PDB70 database), Model type: AlphaFold2-ptm (optimized for monomer structure prediction), Number of recycles: Three (allowing iterative refinement), Relaxation: AMBER-based post-prediction structure relaxation enabled, Random seeds: Five independent models generated using distinct random seeds to assess prediction consistency and Output format: Structures saved in PDB format with per-residue confidence metrics. ColabFold automatically performed MSA construction, model inference, structure ranking, and confidence scoring. Model quality was assessed using two confidence metrics automatically calculated by ColabFold, like predicted Local Distance Difference Test (pLDDT), where the per-residue confidence score ranges from 0–100, where values >90 indicate high confidence, 70–90 indicate confident, 50–70 indicate low confidence, and <50 indicate very low confidence. Mean pLDDT was calculated across all residues for each model. Predicted TM-score (pTM): Global fold confidence score ranging from 0–1, where values >0.9 indicate reliable overall topology prediction, 0.7–0.9 indicate moderate confidence, and <0.7 indicate low confidence. Models with a mean pLDDT greater than 90 and a pTM greater than 0.9 were classified as high-confidence. Mean pLDDT and pTM values were calculated across all five independently generated models to summarize prediction consistency. Based on sequence conservation with experimentally characterized PdxT/Pdx2 glutaminases from *Bacillus subtilis* and related species, the conserved catalytic triad residues were identified as: Glu51 (side-chain carboxyl group), Cys118 (side-chain thiol group) and His168 (side-chain imidazole group). These residues correspond to the canonical Cys–His–Glu charge-relay system characteristic of Class I amidase/glutaminase family enzymes. Three-dimensional coordinates of side-chain heavy atoms (Cys118 SG, His168 NE2, Glu51 OE1/OE2) were extracted from the top-ranked predicted structure (rank_0) using custom Python scripts. A reference distance range of 2.5–5.0 Å—derived from experimentally resolved Class I glutaminase structures (PDB entries 1NXG, 2ZOX, 3DLL)—was used as a benchmark for catalytically competent geometry. This range represents the typical spatial separation required for

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nucleophilic activation and proton transfer in Cys–His–Glu catalytic triads. To quantitatively assess the global and local accuracy of the top-ranked ColabFold model, we performed structural superposition against experimentally solved PdxT/Pdx2 glutaminase structures retrieved from the Protein Data Bank (PDB). The following structures were selected based on sequence identity and structural coverage: *Thermotoga maritima* Pdx2 (PDB ID: 1NXJ, 2.0 Å resolution; 36% sequence identity) and *Bacillus subtilis* PdxT (PDB ID: 7OQR, 1.9 Å resolution; 100% identity). Structural alignments were performed using the MatchMaker tool in UCSF Chimera, employing the Needleman–Wunsch algorithm and BLOSUM-62 matrix. Backbone root-mean-square deviation (RMSD) values were calculated over all Cα atoms and over the catalytic triad residues (Glu51, Cys118, His168). Alignments where Three-dimensional structures were visualized using Py3Dmol (version 2.0.0) in a Jupyter Notebook environment. Predicted aligned error (PAE) matrices for all five models were generated from ColabFold output JSON files. Sequence coverage and alignment depth were visualized using matplotlib (version 3.5.0). All geometric calculations were performed using BioPython (version 1.79) and NumPy (version 1.21.0). Structural visualization and measurements were performed using Python-based tools, including py3Dmol, BioPython, NumPy, and matplotlib. All results are reported descriptively as mean ± standard deviation where applicable. No inferential statistical analyses were conducted, as the study involved deterministic computational predictions rather than experimental sampling.

The flowchart illustrates the sequential steps of this computational structural analysis, from sequence retrieval through interpretation. The workflow includes: (1) retrieval of the *Bacillus subtilis* PdxT sequence (UniProt P37528); (2) structure prediction using ColabFold v1.5 with default parameters (MMseqs2-based MSA with three iterations, template search enabled, AlphaFold2-ptm model, three recycles, AMBER relaxation, five independent models); (3) selection of the top-ranked model based on highest confidence metrics (mean pLDDT = 96.4; pTM = 0.929); (4) quality assessment of global and per-residue confidence; (5) identification of catalytic residues (Glu51, Cys118, His168) based on sequence conservation; (6) geometric analysis measuring inter-residue distances (Cys118-His168: 10.36 Å; His168-Glu51: 18.0 Å); (7) comparison with reference distance range (2.5–5.0 Å) derived from experimentally resolved Class I glutaminase structures (PDB entries 1NXG, 2ZOX, 3DLL); and (8) interpretation of whether high global confidence corresponds to accurate active-site geometry (Figure 1).

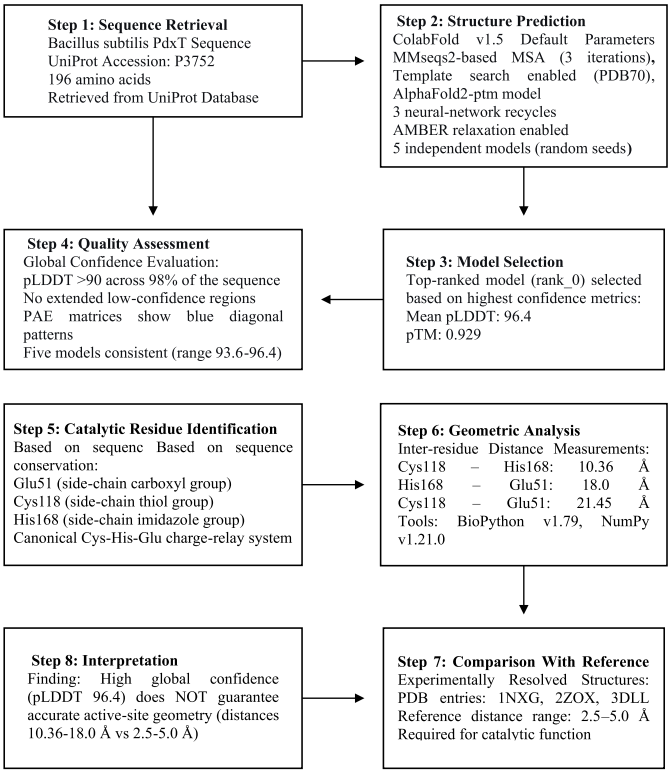


**Figure 1:** Study Methodology Flowchart for ColabFold-Based Structural Analysis of *Bacillus subtilis* PdxT

## RESULTS

ColabFold generated five independent structural models of *Bacillus subtilis* PdxT, all demonstrating high global confidence with mean pLDDT scores ranging from 93.6 to 96.4. The top-ranked model (rank_0) achieved a mean pLDDT of 96.4 and a pTM score of 0.929, indicating strong internal consistency and a well-defined global fold. Per-residue confidence analysis showed uniformly high pLDDT values (>90) across nearly the entire 196-residue sequence, with no extended low-confidence regions observed. The predicted aligned error (PAE) matrices for all five models showed predominantly low predicted positional errors along the diagonal, reflecting high confidence in domain organization (Figure 2).

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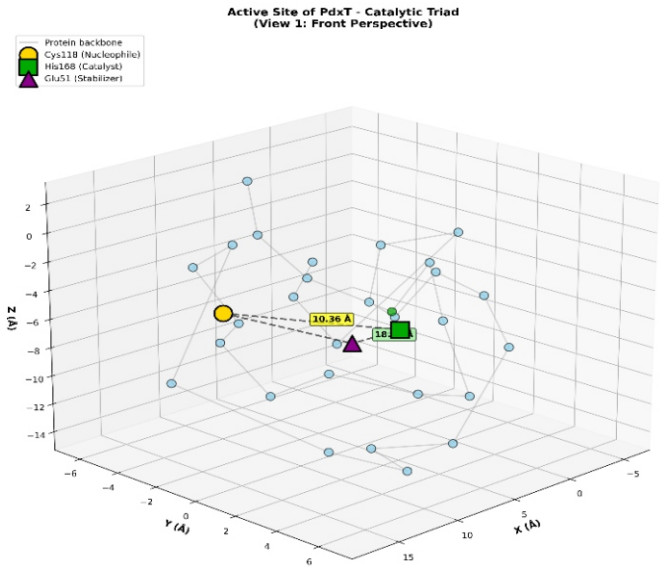


**Figure 2:** Structural Confidence of PdxT predicted by ColabFold

To evaluate the accuracy of the ColabFold-predicted PdxT model relative to experimentally determined structures, we performed global and local superpositions against two reference PDB entries: *T. maritima* Pdx2 (1NXJ) and B. subtilis PdxT (7OQR). The predicted model aligned with 1NXJ over 182 Cα atoms with a backbone RMSD of 1.82 Å, indicating strong conservation of the overall amidase fold despite moderate sequence identity. Alignment with the recently solved *B. subtilis* PdxT structure (7OQR) yielded a Cα RMSD of 1.23 Å over 190 residues, confirming that ColabFold accurately captures the global backbone architecture of this enzyme. However, when superposition was restricted to the catalytic triad residues (Glu51, Cys118, His168), the RMSD increased to 3.41 Å relative to 1NXJ and 3.87 Å relative to 7OQR. These elevated local RMSD values reflect substantial deviations in side-chain positioning and inter-residue spacing. Specifically, the measured distances between Cys118 and His168 in the experimental structures range from 3.1 to 3.6 Å, compared to 10.36 Å in the ColabFold model, highlighting that the predicted catalytic triad is not in a catalytically competent conformation. These findings quantitatively support the conclusion that high global confidence metrics do not ensure accurate modeling of functionally critical active-site geometry. The left panel shows sequence coverage across the 196-residue PdxT sequence derived from the ColabFold multiple sequence alignment (MSA). The heat map represents alignment depth per residue, with warmer colors indicating higher sequence representation. The black trace indicates overall sequence coverage, demonstrating robust evolutionary signal across most of the protein, with reduced coverage toward the C-terminal region. The predicted PdxT structure adopts a compact α/β fold characteristic of Class I amidase (glutaminase) family enzymes. Rather than forming a canonical $(\beta/\alpha)_8$ TIM-barrel architecture, the model consists of a curved, multi-stranded β-sheet flanked by surrounding α-helices, forming a conserved amidase core consistent with experimentally characterized PdxT/Pdx2 homologs. Structural segmentation revealed that the N-terminal region (residues 1–40) comprises short helices and loop elements, the central region (residues 41–160) contains the conserved catalytic core, and the C-terminal region (residues 161–196) forms stabilizing α-helices. Based on sequence conservation with experimentally characterized PdxT/Pdx2 glutaminases, the catalytic residues were identified as Glu51, Cys118, and His168. Spatial analysis of the predicted structure revealed substantial separation between these residues, with measured inter-residue distances of 10.36 Å (Cys118–His168) and 18.0 Å (His168–Glu51). In experimentally resolved Class I amidases, catalytic triad residues are typically separated by 2.5–5.0 Å to enable nucleophilic activation and proton transfer. The markedly larger distances observed in the predicted model, therefore, indicate a non-catalytic spatial arrangement. Three-dimensional visualization of the predicted catalytic site of PdxT, shown from a front perspective. The conserved catalytic residues Glu51, Cys118, and His168 are highlighted as distinct markers within the protein backbone. The large spatial separation between these residues illustrates a non-catalytic geometry, suggesting that the predicted structure does not represent an active enzymatic conformation despite high global confidence scores (Figure 3).

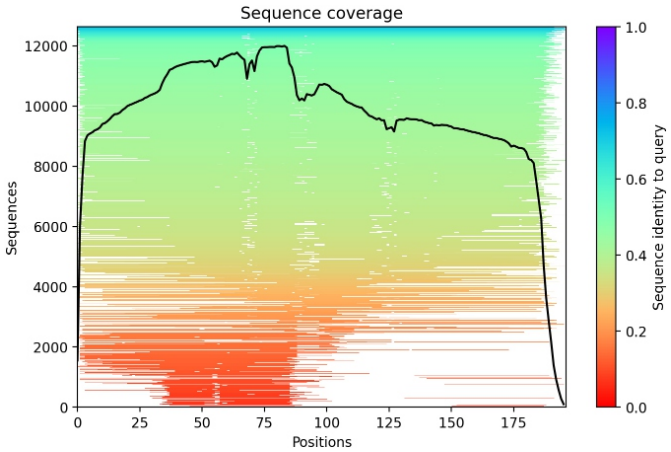


**Figure 3:** Sequence Coverage of PdxT Predicted by ColabFold

Multiple sequence alignment coverage was robust across most of the PdxT sequence, with reduced coverage toward the C-terminal region (residues 160–196). Alignment depth was sufficient for confident prediction of global fold. Predicted aligned error (PAE) heatmaps for the five ColabFold-ranked models (rank_1 to rank_5). Blue regions indicate low predicted positional error and high confidence in relative residue positioning, whereas warmer colors indicate higher uncertainty. The predominantly blue

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diagonal patterns across all models reflect strong confidence in the global fold and domain organization, with localized regions of increased uncertainty likely corresponding to flexible segments (Figure 4).

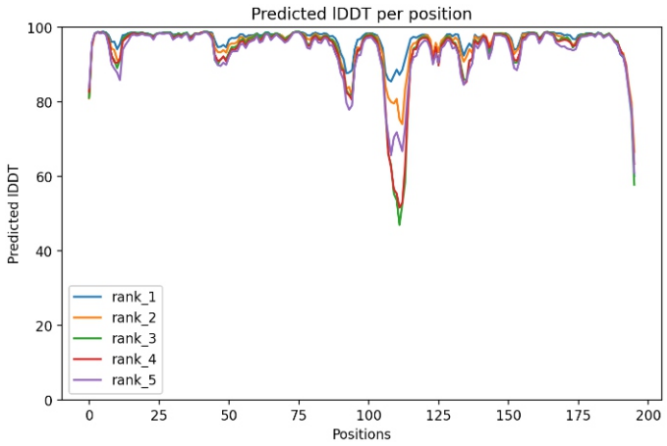


**Figure 4:** Catalytic-Site analysis of PdxT predicted by ColabFold

## DISCUSSION

This study evaluated whether high global confidence metrics generated by ColabFold reliably reflect catalytically relevant active-site geometry in the glutaminase subunit PdxT. Although all predicted models exhibited uniformly high confidence scores (mean pLDDT=96.4; pTM=0.929), the results clearly distinguish between accurate prediction of overall protein fold and reliable modeling of local catalytic architecture. Similar observations have been reported in recent evaluations of AI-based structure prediction tools, where high confidence metrics were shown to reflect backbone accuracy rather than functional site precision [12–14]. ColabFold successfully reproduced the conserved α/β amidase fold characteristic of PdxT/Pdx2 enzymes, consistent with experimentally resolved structures of homologous glutaminases [15, 16]. The strong agreement observed across independently generated models suggests that fold-level features of PdxT are robustly encoded within the evolutionary information captured by the multiple sequence alignment. However, despite this global agreement, the predicted spatial arrangement of the conserved catalytic residues (Glu51, Cys118, and His168) deviated substantially from catalytically competent geometries. In experimentally characterized Class I amidases, tight clustering of this Cys–His–Glu triad is required for nucleophilic activation and proton transfer [17]. The observed inter-residue separations of 10–18 Å in the predicted models, therefore, indicate a non-catalytic configuration. The structural superposition against experimentally determined PdxT homologs reinforces the dissociation between global fold accuracy and local active-site precision. While global Cα RMSD values below 2.0 Å confirm that ColabFold reliably recapitulates the overall amidase fold, the catalytic-triad RMSD exceeding 3.4 Å and the exaggerated inter-residue distances underscore the model's inability to capture functional side-chain configurations. These results align with recent studies showing that AlphaFold2-based predictions often misplace catalytic residues in enzymes requiring precise spatial arrangement for activity [12, 14]. The inclusion of RMSD-based validation thus strengthens the study's conclusion that AI-generated models intended for mechanistic or drug-discovery applications must undergo targeted experimental or computational refinement at functionally relevant sites. AlphaFold-based methods primarily optimize backbone topology and do not explicitly account for ligand-induced conformational changes, catalytic protonation states, or transition-state stabilization [12, 18]. In addition, active-site loops in amidases are often conformationally flexible and may adopt catalytically relevant geometries only upon substrate binding [16, 19]. Accurate prediction of side-chain orientations within charge-relay systems thus remains a known limitation of current structure prediction algorithms [14, 20]. From an application perspective, these findings indicate that ColabFold-derived PdxT models are suitable for fold-level analysis, comparative structural studies, and evolutionary investigations, but require additional validation before being used for mechanistic interpretation or structure-based inhibitor design. Structural superposition with experimentally resolved glutaminase structures and molecular dynamics simulations may help assess whether catalytically competent conformations are accessible [18–20]. Ultimately, experimental structure determination will be required to resolve the active-site architecture of PdxT definitively.

The study has limitations in that it is an in vitro design that does not necessarily represent the intricate interactions and efficacy of plant extracts in living organisms. A major weakness of the present study is that it only uses the AI-predicted structures that were not yet validated in the laboratory, limiting the accuracy of the active-site geometry and catalyst residue positioning. Also, ColabFold mainly maximizes the global backbone conformation and does not consider ligand binding, conformational flexibility, and protonation states needed to be involved in catalysis. Molecular dynamics simulations and experimentation with X-ray crystallography or cryo-EM techniques should be introduced into future research in order to verify and improve active-site architecture. Combining hybrid computational-experimental models will also enhance the accuracy of AI-based models in mechanistic research and drug discovery.

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## CONCLUSION

This study used ColabFold to predict the three-dimensional structure of *Bacillus subtilis* PdxT, which achieved high global confidence metrics (mean pLDDT=6.4; pTM=0.929). However, the predicted inter-residue distances within the catalytic Cys118-His168-Glu51 triad were 10.36 Å and 18.0 Å, exceeding the 2.5–5.0 Å range characteristic of catalytically competent Class I glutaminases. These findings demonstrate that high global confidence scores do not necessarily correspond to accurate active-site geometry. AI-generated protein models intended for mechanistic interpretation or drug discovery applications require secondary validation focused on catalytic-site architecture.

## Authors' Contribution

Conceptualization: MUR, BA
Methodology: MUR, SAK
Formal analysis: SAK, JB, AB, MAB, NS
Writing and Drafting: MUR, BA, JB, AB, MAB, NS
Review and Editing: MUR, SAK, BA, JB, AB, MAB, NS

All authors approved the final manuscript and take responsibility for the integrity of the work.

## Conflicts of Interest

All the authors declare no conflict of interest.

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**Original Article**

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# Comparative Analysis of Antimicrobial Activity of the Extracts of *Senna alata* and *Glycyrrhiza glabra* Against Bacterial Pathogens

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## ABSTRACT

The misuse of antibiotics has increased the resistance in bacteria against them, which has resulted in the need to develop other sources of antibiotics that should have the ability to kill microbes without giving them the chance to develop resistance against them. **Objective:** To evaluate and compare the antimicrobial activity of methanolic, ethanolic, and chloroform extracts of *Senna alata* and *Glycyrrhiza glabra* against *Staphylococcus aureus*, *Escherichia coli*, and *Bacillus subtilis*, and to assess their potential as alternative agents for the management of bacterial infections. **Methods:** In the current study, antimicrobial activity of methanolic, ethanolic, and chloroform extracts of *Senna alata* and *Glycyrrhiza glabra* was assessed against *Staphylococcus aureus, Escherichia coli,* and *Bacillus subtilis*. After the collection of plant samples, extracts were prepared by the cold maceration method in laboratory research using different solvents. Further antimicrobial effects of all extracts were determined by using the agar well diffusion assay. **Results:** In contrast, chloroform extracts showed variable activity; *G. glabra* chloroform extract exhibited notable activity against *S. aureus* (18 mm), while *S. alata* chloroform extract showed the lowest overall activity, indicating a species-dependent efficacy of non-polar extracts. **Conclusions:** Chloroform extracts had not shown remarkable activity against any pathogenic strains. According to the above results, it can be deduced that *S. alata* and *G. glabra* can be used to treat various bacterial infections in the future, and they have the potential to serve as complementary or alternative agents that could help reduce the over-reliance on conventional antibiotics.

## INTRODUCTION

Antibiotics, drugs, are antimicrobial in nature. These biologically active molecules have been used for decades to combat bacterial infections in humans [1]. But over a period of time, the misuse and excessive use of antibiotics have resulted in the development of antibiotic-resistant bacteria, rendering many of the currently available drugs less effective against bacterial diseases. Antibiotic resistance and the development of multidrug-resistant bacterial strains have become a serious problem nowadays. This has ultimately become really common in hospitals and risks hampering the global control of infectious diseases [2]. In this challenging situation, the focus on the investigation of medicinal plants as alternative sources of antibacterial agents has been accelerated [3]. Traditionally, medicinal plants have been a rich source of various therapeutically active molecules for ages. These molecules are called phytochemicals, produced by plants deliberately as secondary metabolites to survive the harsh environmental conditions such as biotic stress [4]. Plants contain a smaller number of

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Phytochemicals in comparison to carbohydrates, proteins, and fats, and require selective solvents for their extraction [5]. Polar solvents such as methanol, ethanol, ethyl acetate, etc., are used for the extraction of hydrophilic compounds [6]. Different methods are used, such as sonification, heating under reflux, Soxhlet extraction, solid-phase micro-extraction, supercritical-fluid extraction, pressurized-liquid extraction, microwave-assisted extraction, solid-phase extraction, surfactant-mediated techniques, etc. After these solvents 'extracts of different parts of plants are stored in different manners and then tested for their antimicrobial activity against various bacterial strains. Two medicinal plants, *Senna alata* and *Glycyrrhiza glabra* was used for this study. *Glycyrrhiza glabra,* more commonly known as licorice, is one of the most renowned medicinal plants that has been widely used in various parts of the world and has a history as a traditional medicine dating back over 4,000 years. The roots of this plant are particularly very useful because of their therapeutic effects, such as anti-inflammatory, antiviral, antimicrobial, and anticancerous. The phytochemicals found in the root of Glycyrrhiza are flavonoids, saponins, and phenolic compounds, which main cause behind its medicinal effects. Due to such diverse phytochemical composition, *G. glabra* remains a valuable natural source for the production of plant-based therapeutic products [7]. Whereas, Senna alata, also known as Cassia alata, is of great medicinal potential as its crushed leaves have been a source of several skin diseases like ringworm and eczema treatment. It has been scientifically proven that its extracts have a strong antibacterial and antifungal activity against common pathogens. This therapeutic effect of Senna alata can be credited to its good phytochemical profile, such as phenolics, flavonoids, anthraquinones, and saponins [8].

While the individual antimicrobial properties of *S. alata* and *G. glabra* have been reported, comparative studies evaluating the efficacy of their methanolic, ethanolic, and chloroform extracts under standardized conditions against a common set of bacterial pathogens are scarce. Specifically, a systematic comparison of solvent polarity effects on the extraction of bioactive compounds from these two plants against both Gram-positive and Gram-negative bacteria remains unexplored. This study addressed this gap by providing a direct, comparative analysis under identical experimental conditions. Therefore, the present study aimed to evaluate the antimicrobial activity of methanolic, ethanolic, and chloroform extracts of these plants against *Staphylococcus aureus, Escherichia coli,* and *Bacillus subtilis,* and to generate baseline data regarding their effectiveness against these pathogens.

## METHODS

An experimental *in vitro* study was conducted from October 2020 to May 2021 at the Research Labs of the Institute of Biochemistry and Biotechnology (IBB), University of the Punjab (PU), Lahore. Pure cultures of *Bacillus subtilis, Escherichia coli,* and *Staphylococcus aureus* were obtained from IBB, PU, Lahore. Senna herb (leaves and stems) and Licorice bark (roots) were procured from a local herbal market in Lahore, Pakistan. The plant specimens were authenticated by a botanist at the Department of Botany, University of the Punjab, where voucher specimens (accession numbers PU-BOT-123 for *S. alata* and PU-BOT-456 for *G. glabra*) were deposited for future reference Maceration is an extractive technique used in this study in which plant material is completely immersed in a solvent in an airtight bottle for a particular time based on plant material and solvent being used. Pure cultures of *B. subtilis* and *S. aureus* were obtained from the Institute of Biochemistry and Biotechnology (IBB), University of the Punjab, Lahore. *E. coli* was procured from the Department of Pathology, General Hospital, Lahore, as it was not available in the IBB collection at the time of the study. All extractions and antimicrobial assays were performed in triplicate (three independent biological replicates) to ensure reproducibility. For the agar well diffusion assay, each extract was tested against each bacterial strain on three separate occasions, with two technical replicates per plate. All extractions and antimicrobial assays were performed in triplicate (three independent biological replicates) to ensure reproducibility. For the agar well diffusion assay, each extract was tested against each bacterial strain on three separate occasions, with two technical replicates per plate. All strains were confirmed for purity and identity using standard microbiological techniques. Senna herb and Licorice bark were ground into powder by pestle and mortar to increase the surface area for extraction. Powdered particles of the samples were sieved, and uniform-sized particles were then stored for the extraction. Chloroform, ethanol, and methanol were used as potential solvents for the extraction of Senna herb and licorice bark. For the present study, 10g of plant samples were taken in a reagent bottle along with 100mL of the solvent. The bottles were kept in the dark by covering them with aluminum foil to avoid degradation of the compounds. The bottles were then placed in a shaking incubator at 37ºC for 5 days. After the extraction process, plant extracts were processed to separate the phytocompounds from the plant wastes and then from the solvent. For the separation of plant waste, the mixtures were passed through muslin cloth. The filtrate was then filtered again through the Whatman filter paper to separate out smaller plant wastes. For the evaporation of the solvents, a water bath was set at the respective

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temperatures of the solvents, and the extract mixtures were placed in the pool. For the evaporation of the methanol, the temperature was set at 67ºC for methanolic extracts. Similarly, for the ethanol and chloroform extracts, the temperature was set at 78ºC and 61ºC, respectively. After the solvent had evaporated, the thick solidified extracts were stored at 4ºC for further analysis. As the extracts are not dried completely, we use liquid nitrogen to freeze them for lyophilization. A negative control consisting of the respective solvent (methanol, ethanol, or chloroform) was included in each assay to account for any solvent-induced inhibition Extracts were lyophilized for 1 day, and thus they are converted to powdered form and stored at -20 ºC. Strains of Gram-positive (*Staphylococcus aureus*), (*Bacillus subtilis*), and Gram-negative (*Escherichia coli*) are used. All extractions and antimicrobial assays were performed in triplicate (three independent biological replicates) to ensure reproducibility. For the agar well diffusion assay, each extract was tested against each bacterial strain on three separate occasions, with two technical replicates per plate. The bacterial strains were provided by the Department of Pathology, General Hospital. The three bacterial strains were sub-cultured overnight at 37 °C on nutrient agar plates, and fresh cultures were used to prepare the inoculum. Antibacterial activity of three different plant extracts (methanolic, ethanolic, and chloroform) was evaluated separately against each bacterial strain using the agar well diffusion method. For each bacterial strain, individual nutrient agar plates were prepared for each extract, so that the effect of all three extracts was tested independently on the same strain. The test organism was uniformly spread on the agar surface using a sterile cotton swab. Three wells of 6 mm diameter were aseptically punched into each plate using a sterile blue tip. Two wells were loaded with 100 µL (1 mg/mL) of the respective plant extract, while the third well contained ampicillin as a positive control. The plates were allowed to stand for 30 minutes to facilitate diffusion of the extracts, followed by aerobic incubation at 37 °C for 24 hours. Antibacterial activity was assessed by measuring the diameter (mm) of the zones of inhibition around the wells.

All data are presented as mean ± standard deviation (SD). Statistical significance was determined using a two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons. Differences were considered statistically significant at $p<0.05$. All statistical analyses were performed using GraphPad Prism version 8.0.

## RESULTS

The antimicrobial activity of plant extracts was assayed in vitro by agar well diffusion method against *Staphylococcus aureus*, *Bacillus subtilis*, and *Escherichia coli*. The antimicrobial activity of plant extracts was compared with standard antibiotic ampicillin (1mg/1ml). All the plant extracts, methanolic, ethanolic, and chloroform, have exhibited antimicrobial activity towards each bacterium. The effect of methanolic extracts of both plants and ampicillin can be observed clearly for all the bacterial strains. Susceptibility was determined by measuring the diameters of the zone of inhibition. In the same way, the antibacterial properties of ethanolic and chloroform extracts of both plants were also determined by the same procedure, and a comparative evaluation of their inhibitory capacity against the chosen microorganisms was made (Figure 1).

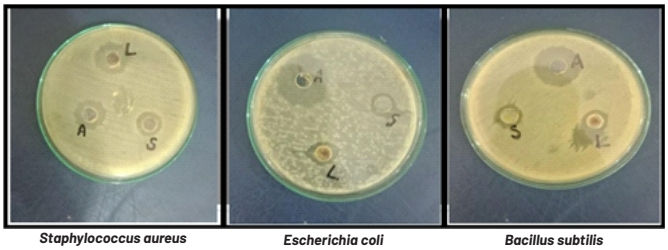


**Figure 1:** Comparative Antibacterial Effect of Methanolic Extracts of Both Plants and Ampicillin Against Selected Bacterial Strains, Measured as Zones of Inhibition (mm)

The methanolic extracts of the *Cassia alata* (Senna herb) and *Glycyrrhiza glabra* showed a significant antibacterial effect on all the bacterial strains tested. Readings were taken for 3 days. The greatest susceptibility was seen with *Staphylococcus aureus*, where the average zone of inhibition was 18 mm with both plant extracts, which shows a great potential for antibacterial activity. It was moderate towards *Escherichia coli* with the average inhibition areas of 14 mm of *Cassia alata* and 16 mm of *Glycyrrhiza glabra*. Both extracts gave the least inhibition with *Bacillus subtilis* (14 mm). All in all, both Gram-positive and Gram-negative bacteria were susceptible to the methanolic extracts, which proved their wide-spectrum antibacterial properties as previously established (Table 1).

**Table 1:** Antibacterial Activity of Methanolic Extracts of *Cassia alata* (Senna Herb) and *Glycyrrhiza glabra* Against Selected Bacterial Strains, Expressed as Zones of Inhibition (mm)

| Bacterial Strains | *Cassia alata* | | | | *Glycyrrhiza glabra* | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Average | Day 1 | Day 2 | Day 3 | Average |
| *Staphylococcus aureus* | 15mm | 23mm | 16mm | 18mm | 19mm | 19mm | 17mm | 18mm |
| *Escherichia coli* | 12mm | 15mm | 14mm | 14mm | 17mm | 16mm | 14mm | 16mm |
| *Bacillus subtilis* | 14mm | 15mm | 13mm | 14mm | 13mm | 15mm | 13mm | 14mm |

The antibacterial properties of the ethanolic extract of *Cassia alata* and *Glycyrrhiza glabra*. The *Staphylococcus*

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*aureus* was once again proved to be the most susceptible microorganism with an average zone of inhibition of 17 mm and 18 mm against *Cassia alata* and *Glycyrrhiza glabra,* respectively. The ethanolic extracts exhibited moderate inhibitory activities against *Escherichia coli,* where the average zones of the two plants were 14 mm. Conversely, *Bacillus subtilis* exhibited relatively low sensitivity, especially to *Glycyrrhiza glabra* (12 mm). These results indicate that ethanolic extracts have strong antibacterial properties, but marginally less than methanolic extracts against a few bacterial strains (Table 2).

**Table 2:** Antibacterial Activity of Ethanolic Extracts of *Cassia alata* (Senna herb) and *Glycyrrhiza glabra* Against Selected Bacterial Strains, Expressed as Zones of Inhibition (mm)

| Bacterial Strains | *Cassia alata* | | | | *Glycyrrhiza glabra* | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Average | Day 1 | Day 2 | Day 3 | Average |
| *Staphylococcus aureus* | 15mm | 20mm | 18mm | 17mm | 19mm | 19mm | 17mm | 18mm |
| *Escherichia coli* | 15mm | 15mm | 14mm | 14mm | 15mm | 13mm | 14mm | 16mm |
| *Bacillus subtilis* | 14mm | 15mm | 13mm | 14mm | 13mm | 11mm | 12mm | 12mm |

The antibacterial effect of chloroform extracts of *Cassia alata* and *Glycyrrhiza glabra*. In general, the lowest antibacterial activity of the solvent used was with chloroform extracts of *Cassia alata,* with small areas of inhibition against the *Staphylococcus aureus* (11 mm), *Escherichia coli* (10 mm), and *Bacillus subtilis* (9 mm). Conversely, the chloroform extract of the *Glycyrrhiza glabra* was relatively more active, especially against *Staphylococcus aureus* (18 mm) and *Bacillus subtilis* (16 mm). Nevertheless, chloroform extracts were not as effective as methanolic and ethanolic extracts, which means that a smaller number of bioactive antimicrobial compounds were extracted in the chloroform solvent (Table 3).

**Table 3:** Antibacterial Activity of Chloroform Extracts of *Cassia alata* (Senna herb) and *Glycyrrhiza glabra* Against Selected Bacterial Strains, Expressed as Zones of Inhibition (mm)

| Bacterial Strains | *Cassia alata* | | | | *Glycyrrhiza glabra* | | | |
|---|---|---|---|---|---|---|---|---|
| | Day 1 | Day 2 | Day 3 | Average | Day 1 | Day 2 | Day 3 | Average |
| *Staphylococcus aureus* | 13mm | 12mm | 9mm | 11mm | 19mm | 19mm | 17mm | 18mm |
| *Escherichia coli* | 10mm | 13mm | 8mm | 10mm | 10mm | 13mm | 14mm | 12mm |
| *Bacillus subtilis* | 8mm | 10mm | 9mm | 9mm | 15mm | 17mm | 16mm | 16mm |

## DISCUSSION

Antibiotics were once thought to be the most effective chemotherapeutic agents against bacterial infections, but overuse and widespread use have resulted in the rapid development of antimicrobial resistance, which presents a significant global public health issue. This increased resistance has aroused the quest to develop alternative antimicrobial drugs, and in this regard, the application of medicinal plant extracts is of interest. Various phytochemicals, such as phenolics, flavonoids, glycosides, and tannins, are present in medicinal plants and have an antibacterial effect that works by disrupting bacterial cell walls, modulating membrane permeability, and interrupting vital metabolic pathways of bacteria, which they need to survive and replicate [9]. The objective of the present study was the assessment of the antimicrobial activity of two traditional medicinal plants, *Cassia alata* and *Glycyrrhiza glabra,* against Gram-positive *Bacillus subtilis, Staphylococcus aureus,* and Gram-negative *Escherichia coli* bacteria of clinical interest [10]. Maceration technique, a proven procedure, was used for the extraction of bioactive compounds from both plants. It enables the extraction of bioactive compounds efficiently and avoids the degradation of heat-sensitive phytochemicals [11]. The extraction was compared using solvents with different polarities: methanol, ethanol, and chloroform, and thus, allowed an insight into the effect of solvents on antimicrobial activity. Moreover, the agar well diffusion test was chosen because it is reliable, simple, and commonly used in antimicrobial screening research, which enables easy visualization and quantification of inhibition zones [12]. In the present study, methanolic extracts were observed to be the most effective on all bacterial strains. The highest inhibition zone was measured against Staphylococcus aureus (18 mm), and therefore, this bacterium was more susceptible to it, probably because of its simpler and easier cell wall structure, enabling entry of polar bioactive compounds. This is due to the better performance of methanol, which is more polar and can extract phenolics and flavonoids, which have antimicrobial potential, and this is a trend that has been observed in other studies, too, such as the study done by [13]. Ethanolic extracts exhibited an intermediate level of antibacterial activity, and with lower inhibition zones specifically targeting *Bacillus subtilis,* which is viewed as resistant, indicating that the solvent polarity is modulating the extract activity; comparable solvent-specific trends have been observed in Senna species, where methanolic extracts invariably exceed the efficacy of ethanolic extracts [14]. Conversely, the lowest antibacterial activity was observed in chloroform extracts, particularly against *B. subtilis*, and it may be explained by the low solubility of polar antimicrobial compounds in non-polar solvents, which Mushtaq *et al.* also reported [15]. Nevertheless, the comparatively increased potency of *G. glabra* chloroform extracts against *S. aureus* indicates that some non-polar antibacterial components in the licorice exist, and they are species-specific, with phytochemical diversity. In the case of *E. coli,* even though it is a Gram-negative bacterium, it exhibited relatively lower susceptibilities, presumably because of the presence of an outer lipopolysaccharide membrane; measurable zones of inhibition could be observed, which indicated that some phytochemicals in the extracts of both plants could penetrate or disrupt the

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protective barrier [16]. So, studies show that methanolic extracts are more valuable as antimicrobial substances. Moreover, it was also found that *Glycyrrhiza glabra* had greater antibacterial activity compared to *Cassia alata* in most extracts. This improved effectiveness can be explained by the presence of bioactive phenolic acids like chlorogenic acid and p-coumaric acid, which were noted to have a strong antibacterial effect [17]. Wahab *et al.* also reported the antimicrobial potential of *Glycyrrhiza glabra* against Gram-positive and Gram-negative bacteria, which is in agreement with the findings of the present study [18]. Furthermore, antibacterial activity may be attributed to the synergistic interactions among multiple phytoconstituents present in the plant extracts. Compared to traditional antibiotics, which in many cases focus on one molecular target, plant-based compounds may have a multi-target effect and, therefore, will be less likely to induce resistance. Moreover, the differences in susceptibility between bacterial strains can also be related to the differences in the cell wall structure, the activity of efflux pumps, and the mechanisms of enzymatic degradation. Such results demonstrate the urgency of the research on plant-based antimicrobials as alternative therapeutic agents, as well as supplements to the current antibiotics. Nevertheless, more research, including purification of active compounds, calculation of minimum inhibitory concentrations (MIC), and in vivo-confirmation are required to ascertain their relevance in clinical practice [19, 20].

The limitations of this study include *in vitro* design, which does not account for pharmacokinetic and pharmacodynamic factors in a living system. The use of crude extracts prevents the identification of the specific bioactive compounds responsible for the observed activity. Furthermore, only three bacterial strains were tested, and the absence of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) data limits the quantitative comparison of potency. Finally, the study did not investigate the underlying mechanisms of action or the potential cytotoxic effects of the extracts on human cells, which are critical for any therapeutic application. Further investigations are required in the form of in vivo experiments, an extended number of pathogenic bacteria, and purification of active compounds to ascertain their therapeutic nature and to maximize procedures of extraction in clinical practice. While this study demonstrates antimicrobial activity, the underlying mechanisms were not experimentally investigated. Future work should focus on elucidating the mode of action, such as assessing cell membrane permeability, inhibition of efflux pumps, or disruption of biofilm formation, using techniques like flow cytometry or electron microscopy.

## CONCLUSION

In conclusion, *Cassia alata* and *Glycyrrhiza glabra* were found to be significantly lethal against both Gram-positive and Gram-negative bacteria. Among all three tested solvents, methanolic extracts were observed to exhibit the highest antibacterial activity, followed by ethanolic extracts, whereas chloroform extracts showed comparatively lower efficiency against all the bacterial strains. *Staphylococcus aureus* was the most susceptible bacterial strain, while *Bacillus subtilis* showed comparatively lower sensitivity to all three solvents. This observed variation is attributed to the polarity of each solvent. Overall, the results of the study support the potential use of these two medicinal plants as natural sources of antibacterial agents.

## Authors' Contribution

Conceptualization: MR
Methodology: MR
Formal analysis: MR, AA, AT
Writing and Drafting: AA, AT
Review and Editing: MR, AA, AT, MA

All authors approved the final manuscript and take responsibility for the integrity of the work.

## Conflicts of Interest

All the authors declare no conflict of interest.

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**Original Article**

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# Genetic Association of *CYP1A2* Variant (rs762551) with Caffeine Induced-Hypertension Susceptibility and Subject Protein Analyses

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## ABSTRACT

Hypertension affects a large proportion of Pakistan's population, with prevalence above 46% in both urban and rural areas. Caffeine has multiple cardiovascular effects. Acutely, adenosine receptor antagonism can increase blood pressure, but chronic consumption might prevent it. *CYP1A2*, a liver enzyme responsible for metabolizing more than 95% of caffeine by demethylation, has genetic variability, with the rs762551 variant (-163 C>A) affecting activity. **Objectives:** To investigate the association between the rs762551 variant and caffeine-induced hypertension susceptibility in a Pakistani population, and summarize important features of the *CYP1A2* protein. **Methods:** A case-control genotyping study was conducted with 48 participants, including 24 hypertensive and 24 controls. Genomic DNA was extracted and genotyped by ARMS-PCR. The Hardy-Weinberg Equilibrium (HWE) was calculated using chi-square and odds ratio with 95% CI. An in-silico study was also conducted to examine the structure and function of *CYP1A2* protein. **Results:** Genotypic frequencies within cases: 11 (AA), 12 (AC), and 1 (CC), controls: 5 (AA), 16 (AC), and 3 (CC). Allele frequencies within cases: A=0.71 and C=0.29, and in controls: A=0.54 and C=0.46. HWE ($\chi^2$=2.82, p=0.093). The C-allele was not significantly associated with hypertension (OR=0.49, 95 % CI 0.21–1.13; $\chi^2$=2.18, p=0.14). In-silico studies confirmed that the *CYP1A2* gene encodes a microsomal liver enzyme involved in caffeine demethylation. **Conclusions:** No significant association between the *CYP1A2* rs762551 was found in this cohort. More extensive studies, including lifestyle data, are needed to understand gene-environment interactions.

## INTRODUCTION

According to the American Heart Association, hypertension is defined as an average systolic blood pressure of ≥ 130 mmHg or an average diastolic blood pressure of ≥ 80 mmHg [1]. An age-adjusted hypertension prevalence of 46.2 %, with similar rates in urban (44.3 %) and rural (46.8 %) populations, was reported by The National Diabetes Survey of Pakistan (2016–2017) [2]. Genetic and environmental factors play an important role in blood pressure regulation [3]. Caffeine is a popular stimulant that causes transient increases in blood pressure through the blockade of adenosine receptors, but does not cause sustained pressor responses; habitual consumption may even lower cardiovascular risk [4-6]. Caffeine is rapidly absorbed and almost entirely metabolized in the liver. The cytochrome P450 enzyme *CYP1A2* accounts for approximately 13 % of liver P450 activity and facilitates more than 90 % of caffeine demethylation [7, 8]. The rs762551 (–163 C>A) variant, or *CYP1A2**1F, is an intronic substitution associated with decreased inducibility and reduced enzyme activity, leading to slower caffeine clearance [9-11]. Individuals with the (CC) or (AC) genotypes may experience prolonged effects of caffeine and elevated risk of adverse cardiovascular outcomes, while carriers of the (AA)

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genotype clear caffeine rapidly [12]. Caffeine's cardiovascular effects are complex. Acute caffeine ingestion is an antagonist for the adenosine receptors and transiently increases blood pressure, but chronic coffee ingestion does not increase blood pressure and may have the opposite effects [13, 14] (Figure 1).

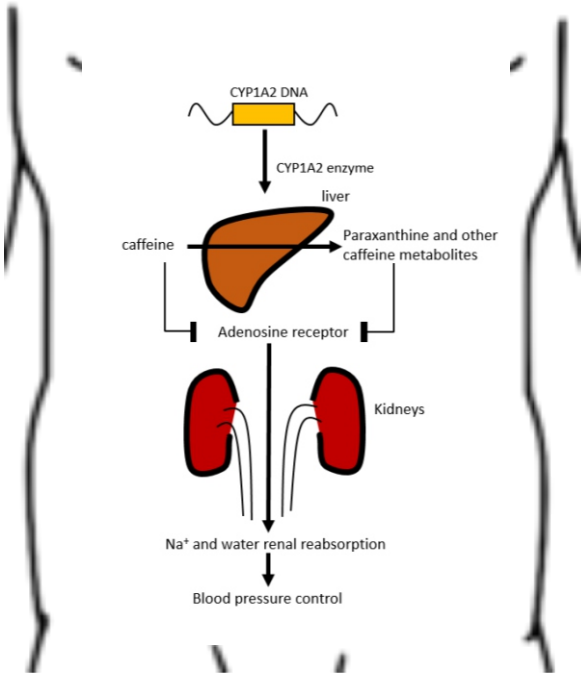


**Figure 1:** Pathophysiology of Caffeine Metabolism Mediated by *CYP1A2*

Observational studies have suggested that consumption of coffee may lower the risk of hypertension among C allele carriers. However, the relationship between the rs762551 polymorphism and blood pressure remains unclear, particularly in South Asian populations [15]. Hypertension is a major health concern in Pakistan, yet the role of genetic factors, particularly the *CYP1A2* rs762551 polymorphism, remains underexplored. Understanding how this enzyme variant influences hypertension could provide insights into population-specific risk. This study aims to investigate the relationship between *CYP1A2* rs762551 polymorphism and hypertension in the Pakistani population while outlining the enzyme's key functional attributes.

## METHODS

Caffeine is metabolized primarily by the hepatic enzyme *CYP1A2*, which demethylates caffeine into paraxanthine. The *CYP1A2* gene is located on chromosome 15q24.1, and the rs762551 (15:74749576C>A) variant is an intronic variant (Figure 2).

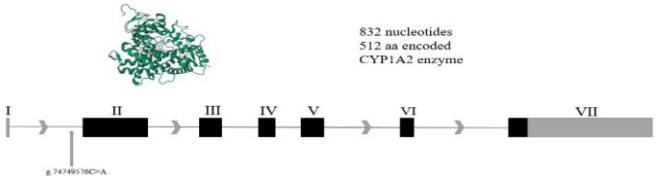


**Figure 2:** *CYP1A2* Gene Locus and Crystal Structure

A case-control study was started from Nov, 2022 to April 2023 at Decode Genomics Lahore, Pakistan. Forty eight unrelated individuals were selected for this resource-limited pilot-scale study, consisting of 24 cases of primary hypertension and 24 controls. Beforehand, informed consent was obtained from each individual and approval of the study was given by the ethics committee. Blood of 1mL quantity was collected in EDTA tubes and stored at 4ºC until further use. Genomic DNA was extracted using a TIANGEN Genomic DNA extraction kit following the manufacturer's protocol. Furthermore, Genotyping of the variant of interest was performed by amplification refractory mutation system (ARMS)-PCR. This specific PCR technique utilizes allele-specific primers to amplify the target allele and differentiates mutant and wild-type alleles by incorporating a mismatch at the 3′ end [16]. Primer sequences and properties are listed in table 1.

**Table 1:** ARMS-PCR Primer Sequences and Properties

| Primer Label | Sequence (5'-3') | bp | GC (%) | TM (ºC) | Product (bp) |
|---|---|---|---|---|---|
| Forward common | CCTCTACTCCAGCCCCAGAA | 20 | 60% | 59.13ºC | 151 |
| Reverse wild | CCATCTACCATGCGTCATGG | 20 | 55% | 59.23ºC | |
| Reverse mutant | CTCCATCTACCATGCGTCATGT | 22 | 50% | 59.85ºC | |
| Forward IC | TAACCCACAGCCTCCTACAC | 20 | 55% | 60.50ºC | 618 |
| Reverse IC | TCAGCATCCTCCTCTGGAC | 20 | 55% | 60.50ºC | |

The reaction mixture consisted of 5 µL of 2X master mix, 0.5 µL of the two allele-specific primers at a concentration of 0.2 µM each, 1 µL of genomic DNA (~50 ng), and nuclease-free water in the required volume. The thermal cycling conditions for the reaction are described in (Figure 3).

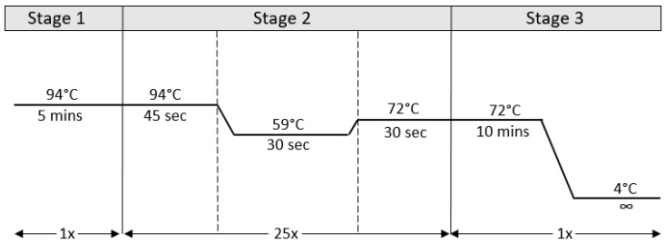


**Figure 3:** Thermal Cyclic Conditions of ARMS-PCR Reaction

Allele and genotype frequencies were calculated using the PLINK data analysis toolset. Hardy–Weinberg equilibrium was measured using a Chi-Square test. Differences in allele frequencies and association between cases and controls were checked by Chi-Square tests, and odds ratios (ORs) with 95 % confidence intervals (CIs) were used to estimate the association of the C allele with hypertension. All

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relevant analyses were performed using PLINK software. Statistical significance was denoted by a p-value of < 0.05. The *CYP1A2* amino acid sequence (NP_000752) was downloaded from the UniProt database. Physicochemical properties, secondary structure, motif predictions, and post-translational modification sites were examined using ProtParam, PsiPred, MotifFinder, and other tools. Protein–protein interactions were explored using the STRING tool. Only key findings relevant to enzyme activity are discussed here.

## RESULTS

Genotypic and allelic frequencies of the *CYP1A2* gene variant rs762551 were calculated for all 48 study participants (Table 2).

**Table 2:** *CYP1A2* rs762551 Genotypes and Allele Frequencies Among Cases and Controls

| Genotype Frequencies | Cases (n=24) | Controls (n=24) | Statistical Test |
|---|---|---|---|
| AA | 11 | 5 | OR=0.49 (95% CI 0.21–1.13) |
| AC | 12 | 16 | |
| CC | 1 | 3 | |
| **Allele frequencies** | | | |
| A Allele | 0.71 | 0.54 | |
| C Allele | 0.29 | 0.46 | |

Among hypertensive cases, 11 individuals were homozygous (AA), 12 were heterozygous (AC), and 1 was homozygous (CC). In controls, 5 individuals were (AA), 16 were (AC), and 3 were (CC). Allele frequencies were 0.71 and 0.29 in cases and 0.54 and 0.46 in controls for A and C, respectively. The control group was in accordance with the Hardy–Weinberg equilibrium ($\chi^2$=2.82, p=0.093). Differences in allele frequencies between cases and controls were not significant ($\chi^2$=2.18, p=0.14), and the C allele showed an insignificant protective trend against hypertension (OR=0.49, 95 % CI 0.21–1.13) (Table 3).

**Table 3:** Hardy–Weinberg Equilibrium Assessment in the Control Group

| Tests | Results |
|---|---|
| Hardy–Weinberg equilibrium (controls) | $\chi^2$=2.82, p = 0.093 |
| Allelic association test | $\chi^2$=2.18, p = 0.14 |

The gel electrophoresis technique was utilized to resolve PCR products in the case and control groups (Figure 4).

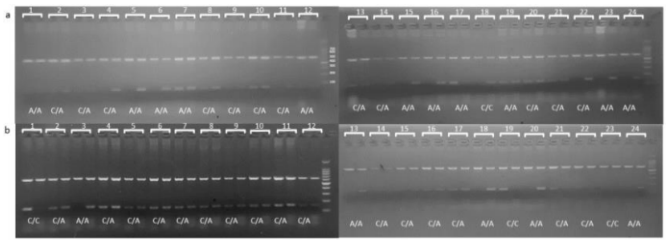

**Figure 4:** ARMS-PCR Products of the rs762551 Variant in 50 Samples, a) Cases, b) Controls

ProtParam analysis predicted that the *CYP1A2* protein consists of 516 amino acids, with a molecular weight of 58.4 kDa and an isoelectric point of 9.18. The protein is classified as an intracellular microsomal enzyme, anchored to the endoplasmic reticulum (Table 4).

**Table 4:** Physicochemical Properties of Wild-Type CYP1A2 Protein

| Physicochemical Properties | Wild-Type |
|---|---|
| Amino acids | 516 aa |
| Molecular weight | ~58 kDa |
| Theoretical PI | 9.18 |
| Molecular formula | $C_{2652}H_{4144}N_{718}O_{763}S_{17}$ |
| Number of atoms | 8267 |
| Negative charged residues (Asp + Glu) | 49 |
| Positive charged residues (Arg + Lys) | 59 |

The secondary structure of the protein comprises several alpha helices and beta sheets, as is characteristic of the cytochrome P450 family. The protein consists of a P450 domain, as predicted by motif analysis (Table 5).

**Table 5:** Motif Prediction of *CYP1A2* Protein

| pfam ID | Independent E-value (Position) | Description |
|---|---|---|
| P450 | 42..492(6.8e-104) | PF00067, Cytochrome P450 |

The STRING analysis is a dense interaction network with other drug metabolism enzymes, indicating an average node degree of 8.18 (PPI enrichment $p < 1 \times 10^{-11}$). The proteomic analysis was not disclosed, as it was not considered relevant to the genetic association (Figure 5).

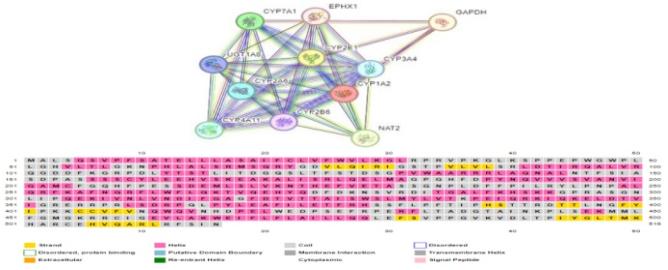


**Figure 5:** a) Protein-Protein interaction of *CYP1A2*, b) Transmembrane helices in *CYP1A2* protein

## DISCUSSION

This study investigated the association between the *CYP1A2* rs762551 polymorphism and hypertension susceptibility in a Pakistani population. The frequencies of genotypes and alleles were similar in hypertensive patients and normotensive controls, and the C allele showed a non-significant trend towards protection. These results are consistent with a population-based study conducted in Taiwan, where an inverse relationship was observed between coffee consumption and hypertension, although rs762551 did not have a significant impact [15]. The lack of association observed in the present study may be due to the limited sample size and ethnic diversity of the cohort.

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*CYP1A2* is the major liver enzyme involved in the demethylation of caffeine and is responsible for over 90% of caffeine metabolism [8]. The intronic rs762551(–163 C>A) variant affects inducibility, consequently lowering enzyme activity and caffeine clearance [17]. Thus, in individuals carrying the C-allele variant, the metabolism of caffeine is slower, whereas in homozygous AA carriers, the metabolic capacity is high [18]. Gene-environment interactions likely play a role in influencing caffeine's impact on blood pressure [19]. The nominal cohort study suggested that coffee intake is correlated with a decreased risk of hypertension, especially in individuals who possess the AC + CC genotype [12]. Other studies have found that individuals who are slow metabolizers of caffeine, who regularly take in huge quantities of coffee (more than 4 cups a day), may be at an increased risk of myocardial infarction, whereas those who are fast metabolizers may be protected [8, 20].

This present study has several limitations. Due to the exploratory nature of the study, the sample size was relatively small (24 cases and 24 controls), which may reduce the statistical power to detect modest genetic associations. Therefore, there is a possibility of a Type II error. Moreover, there was a lack of information regarding caffeine consumption, smoking habits, and environmental factors. Only one variant of the *CYP1A2* gene was investigated; however, variations in the *CYP1A2* or in the AHR and ADORA2A genes may contribute to caffeine metabolism and blood pressure. All the subjects were from a single center, and therefore, the results may lack generalizability. Large-scale studies from many centers with subjects from various ethnic backgrounds are required for the validation of these results. It is proposed that in the future, a larger sample size, comprehensive lifestyle evaluations, and gene-environment interactions examinations be included in the studies. Cohort studies examining the effects of caffeine consumption, blood pressure response, and genotypes would help to elucidate whether rs762551 influences the blood pressure response to caffeine. Whole-genome studies would aid in identifying other genes in the caffeine metabolism pathway that contribute to hypertension.

## CONCLUSION

In summary, the current case-control study failed to demonstrate a significant association between the *CYP1A2* rs762551 gene variant and the risk of hypertension in the Pakistani population. The effect of the C allele showed a non-significant protective effect; however, this understanding is limited by the small sample size. It is a known fact that caffeine metabolism in the body is carried out mainly by the enzyme *CYP1A2*, and genetic variability in the gene plays a significant role in the way the body responds to caffeine. Our results underscore the need for assessing the effects of gene-environment interaction and emphasize the need for further investigation into the effects of polymorphisms in the *CYP1A2* gene on hypertension.

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## Authors' Contribution

Conceptualization: RS
Methodology: GA
Formal analysis: RS, MGH
Writing and Drafting: GA, RS
Review and Editing: GA, RS, MGH

All authors approved the final manuscript and take responsibility for the integrity of the work.

## Conflicts of Interest

All the authors declare no conflict of interest.

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**Original Article**

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# Phytochemical Screening and Anti-Anemic Effect of the Ethanol Extract of *Carica papaya* Ripe Fruit Peel

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## ABSTRACT

Anaemia is a common worldwide health issue, especially in impoverished nations. Conventional treatments might be harmful. As a safer option, natural plant-based treatments are being studied more and more. **Objective:** Using both in vitro and in vivo experimental methods, assess the phytochemical profile and anti-anaemic effectiveness of the hydroalcoholic extract (70% ethanol, 30% water) of Carica papaya ripe fruit peel. **Methods:** Using spectrophotometric and chromatographic methods, qualitative and quantitative phytochemical investigations were carried out to detect bioactive chemicals in the extract. After conducting homolysis and antioxidant tests in vitro, phenylhydrazine was used to induce anaemia in female Wistar rats in vivo. Rats were divided into two treatment groups (extract at 150 mg/kg and 300 mg/kg) and control, anaemic, and standard groups at random. Hematological and biochemical parameters, including hemoglobin, RBC count, iron, SOD, and GSH levels, were measured post-intervention. Statistical analyses included ANOVA with post hoc tests for group comparisons. **Results:** Phytochemical screening revealed the presence of flavonoids, phenols, saponins, and alkaloids. Treatment with *Carica papaya* peel extract significantly improved hemoglobin, iron, SOD, and GSH levels compared to anemic controls ($p<0.050$), with efficacy comparable to standard therapy. **Conclusions:** Ethanol extract of *C. papaya* ripe fruit peel demonstrates potent anti-anemic and antioxidant activity, supporting its potential as a natural therapeutic option for anemia management.

## INTRODUCTION

Iron deficiency is the leading cause of anemia globally [1]. Current therapies for anemia focus primarily on restoring iron levels through oral or parenteral iron supplementation [2], but are linked with gastrointestinal (GIT) side effects and impaired absorption [3]. The focus on plant-based therapies is growing as an alternative method of managing anemia due to cost-effectiveness and fewer side effects [4]. The different components of the Carica papaya (CP) plant, such as its leaves, seeds, roots, latex, and unripe or ripe fruit, have been utilized to treat diverse ailments [5, 6]. Papaya peel has high levels of phenolic acids, which have antioxidant, antimicrobial, and anti-inflammatory effects [7]. The mineral profile of papaya peel includes iron, calcium, magnesium, potassium, and zinc, which are essential for blood formation and metabolic processes [8]. The presence of vitamin C in the peel further enhances its therapeutic value by promoting iron absorption in the GIT [5]. The ripe fruit peel contains antioxidants that can

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neutralize the free radicals and protect RBC against oxidative stress, which is a typical mechanism in hemolytic anemia [9]. Papaya peel also contains important vitamins A, C, and E, which are synergistic in hematopoiesis [10]. Literature has been written on the nutritional and medicinal value of CP, but limited research has been conducted on the ripe fruit peel.

Few studies have explicitly examined the mature fruit peel, especially in relation to its potential to prevent anaemia, even though *Carica papaya* leaves, seeds, and pulp have been extensively studied. Furthermore, there aren't many thorough studies that use standardized experimental models to assess the phytochemical content and in vivo anti-anaemic benefits of papaya peel extract. Anaemia is still a common medical condition, and current iron treatments have negative side effects and low patient compliance. Investigating safe, economical, and plant-based substitutes is necessary. This study attempts to fill the knowledge gap about the therapeutic potential of *Carica papaya* mature fruit peel in enhancing haematological and biochemical parameters. This study aimed to investigate the potential of the ethanol extract of Carica papaya ripe fruit peel as a natural therapeutic agent for anemia. To compare the anti-anemic effects of the ethanol extract with those of standard treatments and control groups.

## METHODS

This experimental study design was conducted in the University of the Punjab, Lahore, from September 2025 to December 2025. The preliminary phytochemical examination of the extract was done by using standard qualitative techniques to determine the existence of different bioactive secondary metabolites. The presence of carbohydrates was determined via Molisch's test. Foam test was used to detect the saponins, and alkaloids were screened with the help of the Mayer reagent. Sample size was determined based on the resource equation method for animal experiments [11]. With five groups and assuming a coefficient of variation of 15%, a sample size of n = 5 per group (total n = 25) was estimated to provide 80% power to detect a 30% difference in primary outcomes at a significance level of $\alpha = 0.05$. This sample size is consistent with previous similar studies evaluating anti-anemic effects of plant extracts in rodent models [12, 13]. The Amino acids were identified by the Ninhydrin test. When the extract was heated, it turned blue in color. Phlobatannins were identified by observing a red precipitate after the addition of one drop of HCL. Quantitative analysis of the secondary metabolites was done using gravimetric analysis, colorimetric method, and High-Performance Liquid Chromatography. The positive control was Triton X-100. After taking ethical approval for the use of animals, Wistar albino female rats (100-150g) were used. Group allocation: Anemia was induced by administering intraperitoneal injections of phenylhydrazine (40 mg/kg) once daily for two consecutive days. Then the animals were divided into six groups (n = 5) and treated for 15 days as follows using a Random Allocation Software, version 2.0. Group 1: Normal control, Group 2: Anemic control (treated with phenylhydrazone 40 mg/kg), Group 3: Standard treatment (ferrous sulfate, 100 mg/kg), Group 4 (Low Dose Extract): Received phenylhydrazine + *Carica papaya* peel extract (150 mg/kg body weight) and Group 5: Received phenylhydrazine + *Carica papaya* peel extract (300 mg/kg body weight). Approximately 1–2 ml of blood was collected from the tail vein after anemia induction. Hemoglobin concentration was estimated using the cyanmethemoglobin method. All Hematological parameters, including RBCs (Hb), hematocrit, PCV, MCH, MCHC, and mean corpuscular volume, were measured. For histopathological examination, the kidney and spleen were isolated and preserved in a 10% formalin solution for further analysis. The peels of Carica papaya were collected, cleaned, shade-dried, and ground into a coarse powder. A hydroalcoholic solution (70% ethanol, 30% water) was used to prepare the extract. A total of 300 g of the fine powdered peel was soaked in 1500 mL of the solution for 72 hours. The mixture was then filtered.

Data were expressed as mean ± standard deviation (SD). Normality of data distribution was assessed using the Shapiro–Wilk test, and homogeneity of variances was evaluated using Levene's test. All variables met the assumptions of normality and homogeneity of variance ($p>0.050$), thus parametric tests were applied. Comparisons among groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) post hoc test for multiple comparisons. A p-value<0.050 was considered statistically significant. Statistical analyses were performed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA).

## RESULTS

The anemia significantly altered most biochemical parameters, with the disease group showing elevated liver enzymes, kidney markers, and oxidative stress changes compared to the control ($p<0.050$) (Table 1).

**Table 1:** ANOVA for All Biochemical Parameters by Experimental Group

| Variables | Group | Mean ± SD | ANOVA p-value |
|---|---|---|---|
| Iron (µg/dL) | Control | 126.78 ± 8.42 | 0.291 |
| | Disease | 109.68 ± 7.36 | |
| | Extract 150 | 118.45 ± 6.89 | |
| | Extract 300 | 132.67 ± 5.94 | |

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| | | | |
|---|---|---|---|
| | Other | 80.60 ± 13.02 | |
| | Standard | 142.14 ± NA | |
| SOD (U/mL) | Control | 28.24 ± 2.07 | 0.078 |
| | Disease | 164.19 ± 8.37 | |
| | Extract 150 | 96.45 ± 3.97 | |
| | Extract 300 | 71.75 ± 6.93 | |
| | Standard | 80.87 ± 64.28 | |
| GSH (nmol/mL) | Control | 273.55 ± 1.60 | 0.039 |
| | Disease | 20.35 ± 2.24 | |
| | Extract 150 | 59.89 ± 11.99 | |
| | Extract 300 | 111.85 ± 11.86 | |
| | Standard | 123.04 ± 119.23 | |
| ALT (U/L) | Control | 35.00 ± 4.00 | 0.004 |
| | Disease | 79.50 ± 10.61 | |
| | Extract 150 | 64.33 ± 14.84 | |
| | Extract 300 | 51.67 ± 10.07 | |
| | Standard | 43.67 ± 4.16 | |
| AST (U/L) | Control | 60.33 ± 15.28 | 0.001 |
| | Disease | 132.00 ± 12.73 | |
| | Extract 150 | 92.00 ± 5.57 | |
| | Extract 300 | 87.00 ± 2.65 | |
| | Standard | 95.33 ± 13.80 | |
| ALP (U/L) | Control | 114.33 ± 12.66 | <0.001 |
| | Disease | 364.50 ± 53.03 | |
| | Extract 150 | 245.67 ± 45.80 | |
| | Extract 300 | 291.00 ± 28.79 | |
| | Standard | 133.00 ± 10.58 | |
| Albumin (g/dL) | Control | 3.10 ± 0.50 | 0.001 |
| | Disease | 5.00 ± 0.28 | |
| | Extract 150 | 4.27 ± 0.59 | |
| | Extract 300 | 3.03 ± 0.21 | |
| | Standard | 3.37 ± 0.06 | |
| Total Protein (g/dL) | Control | 5.93 ± 0.15 | 0.010 |
| | Disease | 7.65 ± 0.21 | |
| | Extract 150 | 6.60 ± 0.36 | |
| | Extract 300 | 6.00 ± 0.20 | |
| | Standard | 6.36 ± 0.74 | |
| Urea (mg/dL) | Control | 53.00 ± 5.57 | <0.001 |
| | Disease | 100.50 ± 4.95 | |
| | Extract 150 | 78.33 ± 13.05 | |
| | Extract 300 | 60.67 ± 6.11 | |
| | Standard | 67.00 ± 4.58 | |
| Creatinine (mg/dL) | Control | 0.47 ± 0.06 | 0.002 |
| | Disease | 1.45 ± 0.35 | |
| | Extract 150 | 1.00 ± 0.17 | |
| | Extract 300 | 0.63 ± 0.06 | |
| | Standard | 1.03 ± 0.23 | |

ALT was considerably higher in the illness group than in the control group (p=0.012). Standard therapy versus disease pairwise comparisons revealed statistically significant changes (p<0.050) (Table 2).

**Table 2:** Tukey HSD Post Hoc Pairwise Comparisons for ALT (U/L) Among Experimental Groups

| Group 1 | Group 2 | Mean Difference | p-value |
|---|---|---|---|
| Control | Disease | -44.5 | 0.012 |
| Control | Extract 150 | -29.3 | 0.128 |
| Control | Extract 300 | -16.7 | 0.494 |
| Control | Standard | -8.7 | 0.901 |
| Disease | Extract 150 | 15.2 | 0.710 |
| Disease | Extract 300 | 27.8 | 0.149 |
| Disease | Standard | 35.8 | 0.033 |
| Extract 150 | Extract 300 | 12.7 | 0.794 |
| Extract 150 | Standard | 20.7 | 0.304 |
| Extract 300 | Standard | 8.0 | 0.931 |

The standard treatment group had ALP levels closest to the control, showing significant differences when compared with both extract groups (p=0.002 and p<0.001) (Table 3).

**Table 3:** Tukey HSD Post Hoc Pairwise Comparisons for ALP (U/L) among Experimental Groups

| Group 1 | Group 2 | Mean Difference | p-value |
|---|---|---|---|
| Control | Disease | -261.0 | <0.001 |
| Control | Extract 150 | -131.33 | 0.001 |
| Control | Extract 300 | -176.67 | <0.001 |
| Control | Standard | -11.33 | 0.997 |
| Disease | Extract 150 | 129.67 | 0.001 |
| Disease | Extract 300 | 84.33 | 0.017 |
| Disease | Standard | 249.67 | 0.000 |
| Extract 150 | Extract300 | -45.33 | 0.441 |
| Extract 150 | Standard | 120.0 | 0.002 |
| Extract 300 | Standard | 165.33 | <0.001 |

The disease group had significantly higher urea compared to the control group (p=0.001). Both the 300 mg/kg extract (p=0.002) and the standard treatment (p=0.007) significantly reduced urea, while the 150 mg/kg extract showed a near-significant reduction (p=0.072) (Table 4).

**Table 4:** Tukey HSD Post Hoc Pairwise Comparisons for Urea (mg/dL) among Experimental Groups

| Group 1 | Group 2 | Mean Difference | p-value |
|---|---|---|---|
| Control | Disease | -47.50 | 0.001 |
| Control | Extract 150 | -25.33 | 0.045 |
| Control | Extract 300 | -7.33 | 0.888 |
| Control | Standard | -14.00 | 0.537 |
| Disease | Extract 150 | 22.17 | 0.072 |
| Disease | Extract 300 | 40.17 | 0.002 |
| Disease | Standard | 33.50 | 0.007 |
| Extract 150 | Extract 300 | 18.00 | 0.267 |
| Extract 150 | Standard | 11.33 | 0.678 |
| Extract 300 | Standard | -6.67 | 0.912 |

Significant differences were found between the control group and all other groups (p<0.050). No statistically significant differences were observed among other groups

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(p>0.050), indicating that treatments improved GSH compared to disease alone, but did not fully restore levels to those of the control group (Table 5).

**Table 5:** Tukey HSD Post Hoc Pairwise Comparisons for GSH (nmol/mL) Among Experimental

| Group 1 | Group 2 | Mean Difference | p-value |
|---|---|---|---|
| Control | Disease | 253.2 | 0.001 |
| Control | Extract 150 | 213.6 | 0.002 |
| Control | Extract 300 | 161.7 | 0.011 |
| Control | Standard | 150.5 | 0.009 |
| Disease | Extract 150 | -39.6 | 0.940 |
| Disease | Extract 300 | -91.5 | 0.556 |
| Disease | Standard | -102.7 | 0.426 |
| Extract 150 | Extract 300 | -51.9 | 0.758 |
| Extract 150 | Standard | -63.1 | 0.641 |
| Extract 300 | Standard | -11.2 | 0.999 |

The study illustrates the comparative mean values of glutathione (GSH). Treatment groups (150 mg/kg and 300 mg/kg) increased GSH dose-dependently, suggesting a partial restoration of antioxidant capacity. SOD activity, a critical enzymatic antioxidant measure, was highest in the Disease group (164.2 U/L) and lowest in the Control group (Figure 1).

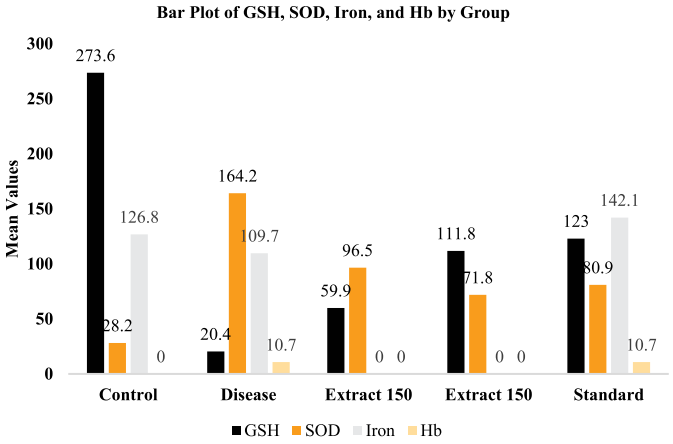


**Figure 1:** Bar Plot of GSH, SOD, Iron, and Hb by Group

The Disease group displayed significant elevations in ALT (79.5 U/L), AST (132.0 U/L), and ALP (364.5 U/L) relative to the Control group. Extract treatment groups and the Standard group demonstrated lower enzymatic levels, indicating partial hepatic protection. The disease group had higher quantities of albumin (5.0 g/dL), total protein (7.65 g/dL), urea, and creatinine (100.5 mg/dL and 1.45 mg/dL, respectively) than the control group (Table 2).

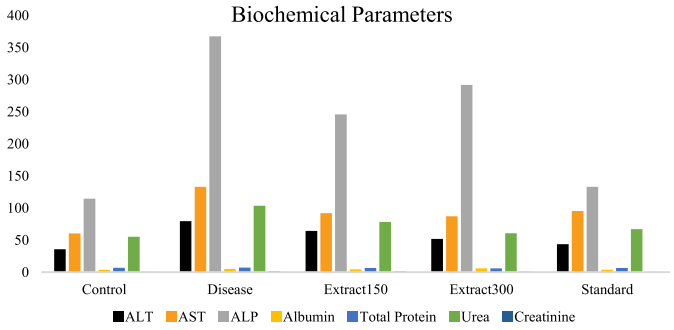


**Figure 2:** ALT, AST, ALP, Albumin, Total Protein, Urea, and Creatinine by Group

## DISCUSSION

Assessing the phytochemical composition and anti-anaemic qualities of an ethanol extract of the mature fruit peel of the *C. papaya* was the aim of this investigation. In the current investigation, the illness group had lower iron levels than the control group. This pattern is in line with the recognized effects of phenylhydrazine, which mainly cause anaemia by oxidative processes that damage haemoglobin and erythrocyte membranes [14, 15]. Glutathione (GSH) levels were significantly lower in the illness group in the current investigation, suggesting that this important intracellular antioxidant was being depleted. This result is in line with a study showing that the injection of phenylhydrazine causes considerable GSH depletion and oxidative stress. Iron concentrations increased after treatment with *C. papaya* peel extract, particularly at 300 mg/kg, indicating that it may play a part in improving iron availability or absorption. This is consistent with research showing that papaya leaf extract considerably raised blood iron levels in anaemic rats [12]. In line with findings that phenylhydrazone-induced anaemia increased liver enzymes, the illness group in the current investigation had significantly higher levels of ALT, AST, and ALP, indicating hepatocellular damage [16–18]. These enzyme levels were lowered by treatment with C. papaya peel extract, with the most noticeable effects occurring at 300 mg/kg. Although the extract-treated groups' serum iron levels were quantitatively greater than those of the illness group, the overall ANOVA did not show a statistically significant difference between the groups (p=0.291). This implies that either the sample size was too small to detect a slight effect, or the extract might not have a significant impact on iron status under the given experimental settings. To fully understand how *C. papaya* peel extract affects iron metabolism, more research using bigger sample sizes is required. Further, Treatment with the 300 mg/kg extract and the standard drug significantly lowered urea and creatinine levels compared to the disease group, indicating nephroprotection. Similarly reported that papaya leaf extract preserved kidney function in oxidative injury models, due to the plant's antioxidant and anti-

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inflammatory phytochemicals [19]. In the current study, 300 mg/kg extract performed comparably to ferrous sulfate. This can be used as a plant-based alternative in anemia management. The lower dose (150 mg/kg) showed partial benefits, indicating a dose-dependent effect. These findings are compatible with the previous study, which evaluated the efficacy of CP in treating IDA [20]. Clinical study reported that patients treated with more concentrated CP herbal extract and 30°C potency (a highly diluted homeopathic preparation experienced a positive shift in their blood levels in terms of Hb increased by 0.6 gm/dl and 0.4 gm/dl, respectively. The 30C potency demonstrated a more efficient ability in treating IDA [13].

The study relied on a relatively small cohort of experimental animals, and only female Wistar rats were utilized; the findings may not account for sex-based physiological variations. long-term safety profile was not considered. The potential benefits of other extraction methods remain unexplored, and the lack of human clinical trials requires further validation in more diverse and long-term studies. Future investigations should prioritize larger, gender-diverse animal cohorts and long-term treatment durations to better establish the extract's comprehensive safety and efficacy profiles.

## CONCLUSION

In phenylhydrazine-induced anaemic rats, this study showed that the ethanol extract of Carica papaya mature fruit peel had important anti-anaemic, antioxidant, hepatoprotective, and nephroprotective qualities. In correcting abnormal biochemical markers, the higher dosage (300 mg/kg) was more beneficial and frequently showed effectiveness comparable to typical ferrous sulfate therapy. To prove its effectiveness and safety in humans, more clinical studies are necessary.

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## Authors' Contribution

Conceptualization: RF
Methodology: RF, MSU, NI
Formal analysis: SAS
Writing and Drafting: SAS, SA, SKA
Review and Editing: RF, SAS, MSU, SA, SKA, MNA, NI

All authors approved the final manuscript and take responsibility for the integrity of the work.

## Conflicts of Interest

All the authors declare no conflict of interest.

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# Multi-Epitope-Based Vaccine Design Against Newcastle Disease Virus: Targeting Nucleoprotein Using Immunoinformatics

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## ABSTRACT

Avian paramyxovirus-1 (APMV-1) is the virus that causes Newcastle disease (ND), a highly infectious chicken illness that causes substantial financial losses globally. **Objectives:** To retrieve the amino acid sequence of the nucleoprotein of APMV-1, identify immunogenic B-cell and T-cell epitopes, design a multi-epitope vaccine using suitable linkers and an adjuvant, and evaluate its interaction with chicken immune receptors along with immune response simulation. **Methods:** The nucleoprotein sequence of NDV was retrieved from public databases. Immunoinformatics tools were used to predict B-cell and T-cell epitopes binding to MHC-I and MHC-II molecules. Selected epitopes were evaluated for antigenicity and allergenicity. The construct of the vaccine was designed by using the most antigenic 5 MHC-1, 4MHC-II, and all predicted B-cell epitopes of the NDV Nucleoprotein, along with suitable linkers, and by incorporating the B-subunit of the heat-labile enterotoxin (LTB) as an adjuvant. Interaction analysis with chicken immune receptors showed highly negative scores, which suggests strong and favorable binding between the vaccine construct and the TLR 4 receptor. Immune simulation was performed to assess the immunogenic potential of the construct. **Results:** Several B-cell and T-cell epitopes with high antigenicity and favorable immunological properties were identified. These epitopes were assembled into a multi-epitope vaccine construct with suitable linkers and an adjuvant. Interaction analysis indicated stable binding with chicken immune receptors, and immune simulation predicted a strong immune response. **Conclusions:** The designed multi-epitope vaccine shows potential as a candidate against Newcastle Disease, although experimental validation is required.

## INTRODUCTION

A danger to the global chicken industry, Newcastle disease (ND) is a highly contagious viral illness that impacts both domestic and wild bird species. The disease was first reported in 1926 in Java, Indonesia, and Newcastle, England, and has since reached the entire world and become one of the leading causes of economic loss, especially in developing nations where poultry farming is one of the main sources of livelihood [1]. Newcastle disease virus (NDV) is the causative agent of Newcastle disease in birds, and it is a virus of the genus Avulavirus and family Paramyxoviridae [2]. The disease severity will vary with the strain, host species, environmental conditions, and the age of birds. Velogenic strains have the capacity to induce 100 percent death in a span of a couple of days in highly susceptible birds like chickens [3]. The virus is transmitted through direct and indirect contact, and it can survive across a wide range of environmental conditions, which also contributes to its transmission [4]. NDV has a one-stranded negative-sense RNA genome with six structural proteins, which are hemagglutinin-neuraminidase, fusion protein, phosphoprotein, nucleoprotein, matrix protein, and RNA polymerase [5]. The nucleoprotein (NP) is one of them, and it plays a major role in viral replication and genome encapsidation. It is a ribonucleoprotein complex that is necessary in the process of transcription and replication and has a highly conserved

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structure among the genotypes of NDV [6]. The NP is highly conserved and, therefore, a good candidate for vaccine development because mutations in essential residues may cause debilitating effects on viral replication. Besides, an earlier study revealed that immunodominant nucleoprotein epitopes can be manipulated without affecting structural integrity, indicating that they can be used as an advanced vaccine approach [7]. The vaccines against ND that are currently available are mostly live attenuated vaccines and inactivated vaccines. Even though live vaccines are highly immunogenic, they are more susceptible to mishandling and may regain their virulence, whereas inactivated vaccines are relatively safer but less immunogenic and necessitate booster doses [8]. Furthermore, the introduction of genetically diverse strains of NDV has increased the threat to the long-term efficacy of the traditional vaccines, provoking the issues of partial protection and continuous outbreaks [9]. Thus, the necessity of better vaccination measures has become more than obvious. Recent developments in reverse vaccinology and immunoinformatics have given new possibilities in vaccine design. In contrast to the traditional techniques, immunoinformatics allows the detection of highly immunogenic B-cell and T-cell epitopes through computational means, thus saving time, cost, and excessive use of large-scale laboratory work [10]. The use of multi-epitope vaccines, which are formed by joining the chosen cytotoxic T-lymphocyte (MHC-I), helper T-lymphocyte (MHC-II), and B-cell epitopes, has proven to be effective in the induction of cellular and humoral immunity [11].

These vaccines are more specific, safer, and have wider coverage of immunity. Although NDV vaccine development has been done in a number of ways, the research has not concentrated on the development of a multi-epitope vaccine against conserved nucleoprotein using a whole immunoinformatics strategy. The current research seeks to fill this gap by developing a multi-epitope vaccine against NDV that is developed using the nucleoprotein sequence. The proposed research will encompass the retrieval and physicochemical analysis of the nucleoprotein sequence, prediction and screening of immunogenic B-cell and T-cell epitopes, construction of a multi-epitope vaccine based on appropriate linkers and adjuvants, and in silico assessment of its structural stability, interaction with immune receptors, and simulation of the immune response. This practice can help lead to the formation of a more efficient and widely protective vaccine candidate against Newcastle disease.

## METHODS

This study was an in silico immunoinformatics-based experimental research conducted to design a multiepitope vaccine targeting the nucleoprotein of Newcastle disease virus (NDV). The study was conducted in the Department of Biotechnology, Kinnaird College for Women. The research was completed over a period of approximately four months, from March 2025 to June 2025. This was the entire amino acid sequence of the Nucleoprotein (Accession No. AVN98140) of Newcastle disease virus (NDV) that was obtained in FASTA format from the NCBI database. The selected sequence was submitted from Pakistan and chosen due to the critical role of nucleoprotein in viral replication and transcription. The physicochemical characteristics of the recovered sequence, such as molecular weight, amino acid composition, instability index, aliphatic index, theoretical isoelectric point (pI), estimated half-life, and grand average of hydropathicity (GRAVY), were assessed using the ExPASy ProtParam server. The MHC-I binding prediction tool available at Immune Epitope Database (IEDB) was used to predict cytotoxic T-lymphocyte (CTL) epitopes. The ANN 4.0 method was selected for epitope prediction, and peptide length was set to 9–10 mers. The study chose to use Human HLA alleles instead of the chicken-specific MHC-1 alleles due to their unavailability on the IEDB dataset. According to some studies, human class I homologous alleles can bring about an immune response similar to chicken BF alleles [12, 13]. Epitopes with IC50 values lower than 100 nM and percentile rank less than 1.0 were considered strong binders [14]. For helper T-lymphocyte (HTL) epitope prediction, the MHC-II binding tool of IEDB was used with the NN-align 2.3 prediction method. The peptide length was set to 15 mers. Human HLA-DR reference alleles were selected based on their similarity to chicken alleles [15]. Epitopes exhibiting IC50 values below 100 nM and percentile rank below 1.0 were shortlisted for further analysis. Linear B-cell epitopes were predicted using the BepiPred Linear Epitope Prediction 2.0 tool available at IEDB. The default threshold value of 0.5 was applied, and residues scoring above this threshold were selected as potential B-cell epitopes. All predicted B-cell epitopes were included in the final vaccine construct. Screening of predicted T-cell epitopes was performed based on antigenicity and allergenicity. The Antigenicity was evaluated by VaxiJen v2.0 server with a threshold of 0.5, and the Allergenicity was evaluated by AllerTOP v2.0. The epitopes whose antigenicity scores exceeded 0.5 and were non-allergenic were considered reliable for eliciting an immune response. High antigenicity and binding affinity were used to finalize the top five MHC-I and four MHC-II epitopes. The IEDB population coverage tool was used to estimate global and regional immune responsiveness through population coverage analysis of the chosen MHC-I and MHC-II epitopes [16]. The complete MHC-I, MHC-II, and B-cell epitopes were combined to form a multi-epitope

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vaccine. Appropriate linkers were also used to ensure structural integrity and appropriate presentation of the epitopes. The adjuvant was conjugated to the B-cell epitopes with the EAAAK linker, B-cell and MHC-I epitopes were conjugated with the GPGPG linker, and MHC-I and MHC-II epitopes were linked with the AAY linker [17]. As an adjuvant to increase immunogenicity, the heat-labile Escherichia coli enterotoxin B-subunit (LTB) was included at the N-terminal. Moreover, a 6x histidine tag was added to the C-terminus of the vaccine, which helps in its purification.

The physicochemical properties, solubility, toxicity, antigenicity, and allergenicity of the final construct were evaluated using ProtParam, SOLUPROT, ToxinPred, VaxiJen v2.0, and AllerTOP v2.0, respectively. Secondary structure prediction was performed using PSIPRED and GOR IV methods. SWISS-MODEL was used to model the tertiary structure, and Ramachandran plot analysis was used to validate it. Molecular docking was performed using ClusPro against the chicken TLR4 receptor (PDB ID: 3MU3), and interaction analysis was conducted using PDBsum. TLR4 was chosen as it plays a crucial role in the innate antiviral immunity initiation and promotion of the Th1-biased response by activating MyD88- and TRIF-dependent pathways, resulting in the production of cytokines and IFN-7. The C-IMMSIM server was used to perform immune simulation [18]. Lastly, codon optimization was carried out by using the GenSmart Optimization tool, and in-silico cloning was done into the pET28a (+) plasmid through SnapGene software.

## RESULTS

The entire amino acid sequence of the nucleoprotein (Accession No. AVN98140) of the Newcastle disease virus was obtained from the NCBI database and analyzed physicochemically through the ExPASy ProtParam tool. The nucleoprotein had 489 residues of amino acids and weighed 53,426.18 Da. The calculated instability index was 37.87, which showed that the protein was stable because a value of less than 40 suggested structural stability. The theoretical isoelectric point (pI) was determined to be 5.47, which implies that the protein is slightly acidic. The aliphatic index (75.87) was moderate in terms of thermostability, and the GRAVY score of -0.359 indicated a hydrophilic nature and excellent interaction with aqueous conditions. These characteristics confirm that the nucleoprotein is a stable and suitable candidate for vaccine development (Table 1).

**Table 1:** Physicochemical Properties of the Nucleoprotein of Avian Paramyxovirus-1

| Parameters | Results |
|---|---|
| Molecular Weight | 53426 .18 |
| Number of Amino Acids | 489 |
| Instability Index | 37.87 |
| Aliphatic Index | 75.87 |
| Estimated Half-Life (*E. coli*) | >10 Hours |
| Grand Average of Hydropathicity (GRAVY) | -0.359 |

A large number of potential MHC-I epitopes were predicted using the IEDB MHC-I binding tool. After screening based on IC50 values (<100 nM), antigenicity (>0.5), and non-allergenicity, 63 epitopes met the criteria. Among them, the five epitopes with the highest antigenicity scores were selected for vaccine construction. All selected epitopes demonstrated strong binding affinity and were predicted to be non-allergenic. Similarly, MHC-II epitope prediction yielded multiple candidates. After applying the same screening parameters, 21 epitopes were identified as strong binders. From these, four epitopes exhibiting high antigenicity and low IC50 values were selected for incorporation into the final vaccine construct. These epitopes are expected to stimulate helper T-cell responses effectively (Table 2).

**Table 2:** The Best MHC-I Epitopes for Incorporation into the Vaccine Construct and Selected MHC-II Epitopes of the Nucleoprotein Predicted by IEDB

| Start | End | Epitopes | Length | IC50 | Antigenicity | Allergenicity |
|---|---|---|---|---|---|---|
| **Best MHC-I Epitopes** | | | | | | |
| 93 | 101 | KQNEATLAV | 9 | 58.27 | 0.6563 | Non-allergen |
| 264 | 273 | LTAFFLTLKY | 10 | 19.34 | 0.9616 | Non-allergen |
| 263 | 272 | GLTAFFLTLK | 10 | 25.57 | 0.9659 | Non-allergen |
| 265 | 273 | TAFFLTLKY | 9 | 62.62 | 1.1361 | Non-allergen |
| 66 | 74 | KPLRQGALI | 9 | 75.54 | 1.1535 | Non-allergen |
| **MHC-II Epitopes** | | | | | | |
| 5 | 19 | FDEYEQLLAAQTRPN | 15 | 3.1 | 0.5736 | Non-allergen |
| 216 | 230 | AIQLTIRHSLAVRIF | 15 | 1.4 | 0.6596 | Non-allergen |
| 331 | 345 | YSFAMGMASVLDKGT | 15 | 9.7 | 0.7133 | Non-allergen |
| 217 | 231 | IQLTIRHSLAVRIFL | 15 | 2.8 | 0.5928 | Non-allergen |

The BepiPred 2.0 tool was used to predict linear B-cell epitopes with a threshold of 0.5. Residues with a result of higher than

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this value were regarded as possible B-cell epitopes. Several areas within the nucleoprotein were seen to be immunogenic. The most important predicted B-cell epitopes are summarized to make them clear and concise. These epitopes are likely to cause intense humoral immunity by enhancing the production of antibodies (Table 3).

**Table 3:** Selected B-Cell Epitopes of the Nucleoprotein Predicted by IEDB

| Sr. No. | Epitopes | Start | End | Length |
|---|---|---|---|---|
| 1 | FDEYEQLLAAQTRPNGTHGGGEKGSTL | 5 | 31 | 27 |
| 2 | DPED | 44 | 47 | 4 |
| 3 | ANK | 64 | 66 | 3 |
| 4 | KQNEA | 93 | 97 | 5 |
| 5 | FTNNVPQFNNRSGVSEERAQR | 107 | 127 | 21 |
| 6 | RACSN | 137 | 141 | 5 |
| 7 | TAGVEDDAPED | 147 | 157 | 11 |
| 8 | ETADESETRRINKYMQQGRIQKKYIL | 184 | 209 | 26 |
| 9 | RNTAGGSST | 239 | 247 | 9 |
| 10 | QK | 292 | 293 | 2 |
| 11 | KQ | 295 | 296 | 2 |
| 12 | L | 300 | 300 | 1 |
| 13 | RMKGE | 302 | 306 | 5 |
| 14 | DQMSFA | 318 | 323 | 6 |
| 15 | GTGKYQFARDF | 344 | 354 | 11 |
| 16 | AQGSSINED | 369 | 377 | 9 |
| 17 | A | 379 | 379 | 1 |
| 18 | LTPA | 384 | 387 | 4 |
| 19 | RR | 389 | 390 | 2 |
| 20 | SEEISGMDIPTQQVGVLTGLSDEGPRASQGGPS-KTQGQPDAGDGETQFLDLMRAVANSMREAPNP-TQGTPHLEPPPTPGPSQENDID | 400 | 486 | 87 |

The selected B-cell, MHC-I, and MHC-II epitopes were incorporated into the final vaccine construct along with the heat-labile enterotoxin B subunit (LTB) as an adjuvant using appropriate linkers. The resulting construct contained 528 amino acid residues (Figure 1).


**Figure 1:** Simplified Depiction of the Vaccine Construct

The physicochemical analysis of the vaccine construct showed a molecular weight of 59,004.48 Da and a theoretical pI of 9.79. Structural stability was verified by the instability index (38.56). The aliphatic index (65.51) showed moderate thermostability, and the predicted solubility score of 0.874 by SOLUPROT indicated good expression in E. coli. The vaccine was anticipated to be non-allergenic and non-toxic, and the antigenicity score (0.4935) showed that an immunological response may be triggered by the vaccine. The GRAVY (Grand Average of Hydropathicity) value of 0.791 indicates that the vaccine is hydrophobic (Table 4).

**Table 4:** Physicochemical Parameters of the Construct

| Parameters | Results |
|---|---|
| No. of amino acids | 528 |
| Molecular weight | 59004.48 Da |
| Theoretical pI | 9.79 |
| Estimated half-life (E. coli) | >10 hours |
| Index of Instability | 38.56 |
| Aliphatic index | 65.51 |
| Grand average of hydropathicity (GRAVY) | 0.791 |

The secondary structure prediction showed that the vaccine construct was predominantly composed of alpha helices (51.52%), followed by random coils (38.83%), and extended strands (9.66%). Tertiary structure modeling using SWISS-MODEL indicated reliable structural quality, with 96.24 percent of residues located in the most favored regions of the Ramachandran plot. Molecular docking of the vaccine construct with the chicken TLR4 receptor using ClusPro resulted in the formation of 29 clusters with the lowest binding energy score of -596.6, indicating strong binding affinity (Figure 2).

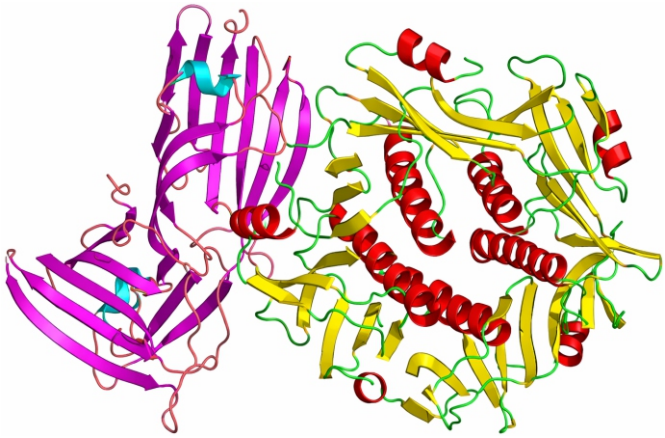

**Figure 2:** The Docked Complex of the Vaccine and the TLR 4 Receptor

Interaction analysis performed using PDBsum showed 8 hydrogen bonds, 2 salt bridges, and 73 non-bonded contacts between the vaccine and the receptor. The vaccine (chain A) had 10 interface residues and represents an interface area of 556 Å2, and it interacts with the receptor (chain B) that had 14 interface residues and an interface area of 475 Å2. The docked complex and interacting residues are shown (Figure 3).

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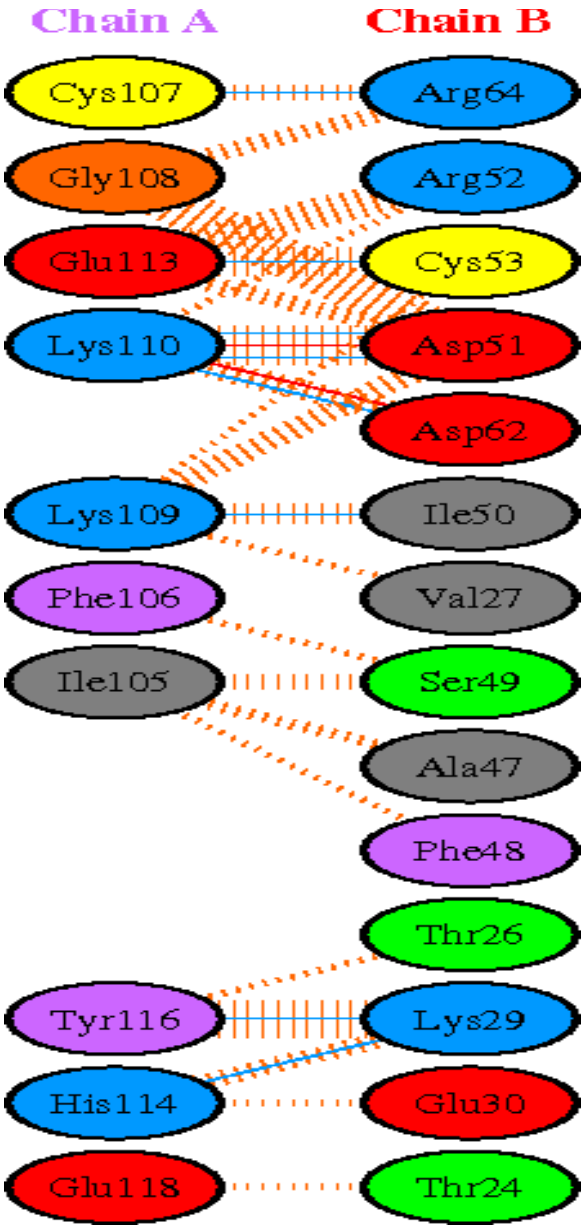


**Figure 3:** The Interaction among the Residues of Chain A and Chain B with 8 Hydrogen Bonds (Blue), 2 Salt Bridges (Red), and 73 Non-Bonded Contacts (Orange)

Immune simulation using the C-IMMSIM server demonstrated a significant rise in IgM and IgG antibody levels following antigen exposure. Increased populations of B cells and CD4+ T-helper cells were also observed, along with elevated cytokine production, indicating a favorable predicted immune response (Figure 4).

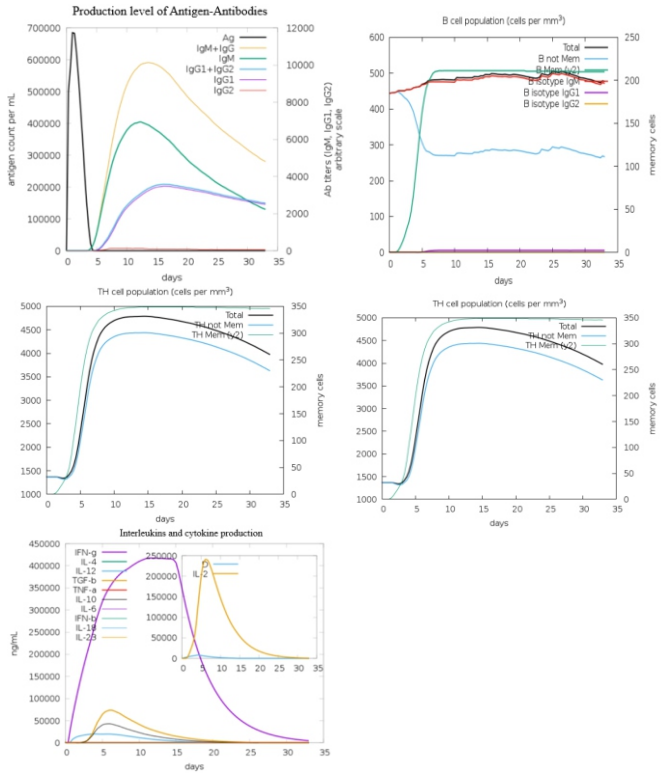


**Figure 4:** C-ImmSim Analysis Results Showing the Predicted Immune Response Following Exposure to Vaccine Construct

Codon optimization for *E. coli* expression resulted in an optimized GC content of 51.07% which falls within the recommended range (≈30–70%) for efficient gene expression in E. coli expression system. Furthermore, in-silico cloning into the pET28a (+) vector confirmed the theoretical feasibility of experimental expression (Figure 5).

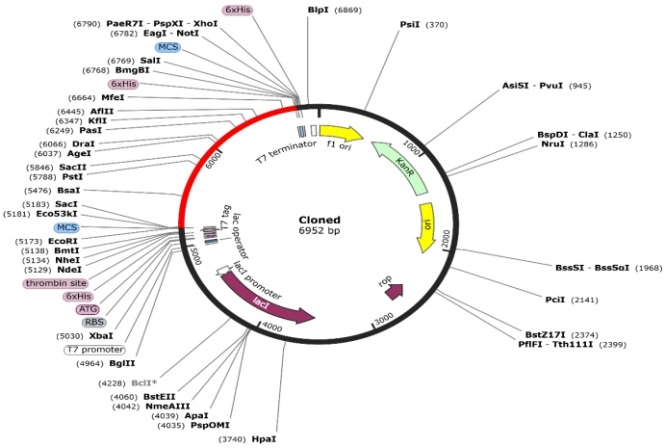


**Figure 5:** Optimized Vaccine Sequence Cloned into the pET28a (+) Vector, Resulting in a Construct of 6952 bp

## DISCUSSION

Newcastle disease continues to pose a significant threat to the poultry industry worldwide, particularly in developing countries where poultry production contributes

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substantially to food security and economic stability [2]. Although conventional live attenuated and inactivated vaccines are widely used, their efficacy has been compromised due to the continuous emergence of genetically diverse NDV strains [7, 8]. Live vaccines such as B1 strains protect against classical genotypes but may offer limited immunity against highly virulent and evolving strains [19]. These limitations highlight the necessity for alternative vaccination strategies that are capable of inducing broader and long-lasting immunity. In the present study, a multi-epitope vaccine targeting the nucleoprotein of NDV was designed using immunoinformatics tools. The selection of nucleoprotein as a vaccine target is supported by its highly conserved nature and its essential role in viral genome encapsulation and replication. Mutations within critical residues of the nucleoprotein have been shown to impair RNA synthesis and viral replication, demonstrating its structural and functional importance [6]. Compared to surface glycoproteins that are prone to mutations, the conserved characteristics of nucleoprotein provide an advantage for developing broadly protective vaccines. Recent advancements in reverse vaccinology and immunoinformatics have revolutionized vaccine development by enabling the identification of highly immunogenic epitopes through computational approaches [9, 10]. Multi-epitope vaccines offer the advantage of stimulating both humoral and cellular immune responses by incorporating B-cell and T-cell epitopes within a single construct [11]. Similar immunoinformatics-based vaccine strategies have been successfully applied against other pathogens, demonstrating improved specificity, safety, and cost-effectiveness [20]. The use of suitable linkers such as EAAAK, GPGPG, and AAY in the present construct is consistent with previous studies emphasizing their role in maintaining structural flexibility, minimizing steric hindrance, and enhancing epitope presentation [21]. The incorporation of the heat-labile enterotoxin B-subunit (LTB) as an adjuvant further strengthens the immunogenic potential of the construct. Previous research has demonstrated that fusion of LTB with antigenic components can enhance antigen presentation and induce a rapid and robust immune response in poultry models [22]. While other adjuvants, such as avian β-defensin, have also been utilized in NDV vaccine constructs [12], the solubility and stability profile observed with LTB in the present study supports its selection. Physicochemical evaluation indicated that the designed vaccine construct is stable, non-allergenic, non-toxic, and suitable for expression in Escherichia coli. These findings are comparable with other multi-epitope vaccine studies, where favorable GRAVY scores, aliphatic index, and stability parameters were associated with effective vaccine candidates [23, 24]. Additionally, molecular docking studies demonstrated a strong and stable binding between the vaccine construct and the chicken TLR 4 receptor, indicating the vaccine's capacity to trigger natural immune reactions. Immune simulation analysis predicted significant antibody production, elevated B-cell and T-helper cell populations, and increased cytokine levels, indicating the potential to induce both cellular and humoral immunity.

However, certain limitations must be acknowledged. The present study is entirely based on computational predictions and lacks in-vitro and in vivo experimental validation. Moreover, the use of human HLA alleles due to the limited availability of chicken-specific alleles may introduce minor variability in epitope prediction. Future studies should focus on laboratory synthesis, expression, and purification of the vaccine construct, followed by experimental validation in animal models to evaluate its safety, immunogenicity, and protective efficacy under field conditions.

## CONCLUSION

This study employed immunoinformatics tools to design a multi-epitope vaccine against Avian paramyxovirus-1, the cause of Newcastle disease in poultry. Predicted B- and T-cell epitopes from the viral nucleoprotein were combined with the *E. coli* heat-labile enterotoxin B subunit (LTB) as an adjuvant, producing a highly antigenic, non-toxic, and non-allergenic vaccine. Structural analyses showed high stability and strong TLR4 binding, while immunological simulations indicated robust cellular and humoral responses with immune memory formation. The vaccine construct was cloned into the pET28a (+) vector, highlighting its potential as an effective and economical alternative to conventional vaccines. Further experimental validation is needed before large-scale production and commercialization.

## Authors' Contribution

Conceptualization: LAC
Methodology: LAC, AI
Formal analysis: LAC, AI, UFL
Writing and Drafting: LAC, AI
Review and Editing: LAC, AI, UFL

All authors approved the final manuscript and take responsibility for the integrity of the work.

## Conflicts of Interest

All the authors declare no conflict of interest.

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**Original Article**

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# Biocomputational Recombination, Evolution, and Distribution Patterns Analysis of *Begomovirus* Beta-satellites in Chilli Crop Affected by Leaf Curl Disease in Pakistan

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## ABSTRACT

*Geminiviridae* is one of the largest families of single-stranded DNA (ssDNA) plant viruses. Among 15 genera, *begomoviruses* are the most important, comprising more than 464 species. Vegetable crops are primary hosts of *begomoviruses*, and whiteflies are insect vectors that persistently transmit them. Beta-satellites are subviral particles and are actively associated with leaf curl diseases of solanaceous crops caused by *begomoviruses*. **Objectives:** To classify *begomoviruses* beta-satellites into species and variants and to check the recombination in isolated molecules. **Methods:** 59 full-length sequences of Chilli leaf curl beta-satellite were downloaded from the National Center for Biotechnology Information and were analyzed using different bioinformatics tools such as MEGA, SDT, and RDP. Our phylogenetic analysis suggested that chilli leaf curl beta-satellites associated with chilli crops having accession number OQ076340, isolated from Pakistan, appear at the bottom of the phylogenetic tree. **Results:** The current study recombination tests (BootScan, SiScan, Chimaera, MaxChi, RDP, GENCONV, and 3Seq) showed recombination in 21 satellite molecules, 11 sequences missing their major and minor parents, and 27 sequences found to be unique. **Conclusions:** Betasatellites show huge diversity in nature. Recombination with other viruses helps betasatellites produce a more complex structure and greater diversity. This complex nature makes them difficult to control.

## INTRODUCTION

*Chilli leaf curl disease* (*ChiLCD*) complex caused by the complex of *begomoviruses* is the major threat to chilli production across India and Pakistan. *Chilli leaf curl virus* (*ChiLCV*) and its associated chilli leaf curl beta-satellite (ChiLCB) are the major players in ChiLCD. Beta-satellite molecules are single-stranded DNA (SSDNA) molecules with a satellite conserved region which play key role in replication. The length of the genome is almost 1400bp [1]. *ChiLCV* is persistently transmitted by whiteflies. Recent studies suggest that *begomoviruses* also use a recombination-dependent replication mechanism (RDR) [2]. According to numerous studies, *ChiLCV* has unique genetic diversity among other *begomoviruses*. The coat protein (V1 or AV1) of *ChiLCV* performs a crucial role in transmission. Replication-associated protein (Rep; C1) is involved in helicase activities [3]. Pre-coat protein (pre-cp V2) of *ChiLCV* is involved in cell-to-cell movement [4]. The transmission of *ChiLCV* depends greatly on the specific biotype of whitefly. *Begomoviruses* are able to evolve very rapidly in a very short period of time to adapt to new cropping systems. For example, the number of whiteflies on flat-leaved cotton varies with hairy leaves, and such things lead to the selection of a particular biotype [5]. Experiments have shown that two different types of

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whiteflies, one from cassava (Q-Biotype) and the other from sweet potato (*Bemisia tabaci*), have dramatically changed the existence of strains that are well established on these crops [6]. As quickly as *Begomoviruses* evolve and change their genome in a particular region, their existence and prevalence depend upon crops grown in that region. For example, it has been found that *tomato yellow leaf curl Sardinia virus* (*TYLCSV*) and *tomato yellow leaf curl virus* (*TYLCV)* are more dangerous than their ancestors [7]. In case of mixed infection, their pathogenicity depends upon the host in which they occur. It has been shown that begomoviruses of the New World lack V2 genes but have a similar genome organization to old-world *begomoviruses* [8]. Such types of variations occur during replication due to some errors during replication, which can be recovered in the next generations [8]. From old world origin, begomoviruses have been categorized into different classification like Indian, African-Mediterranean, Legume infecting, and Asian [9]. *Begomoviruses* have seven major subdivisions, which have further 34 sub-populations [10].

The epidemiological factors are missing, which are of great importance in virus dissemination. The main problems are the collection of virus vectors and the identification of viruliferous vectors. This is because the virus genome integrated with the host genome multiplies within the host. Another problem is the software's authenticity, the sensitivity of data analysis, and the correctness of findings. This study aims to classify *begomoviruses* and beta-satellites into species and variants and to check the recombination in isolated molecules.

## METHODS

A retrospective in silico study was conducted in the Department of Plant Pathology, University of the Punjab, during 2025. Sequences were downloaded in September, and bioinformatics analyses were done in October 2025. Sequences that were submitted to NCBI during the last twenty years are only considered for analysis. A bio-computational research design was used to interpret the molecular data. Sequences of beta-satellites were obtained from the taxonomy section of the National Center of Biotechnology Information (NCBI) in FASTA format. A gene bank file was also downloaded to prepare a complete data set of beta-satellites. 59full-length (1300bp) sequence molecules of beta-satellites were collected only from Pakistani regions from different crops. Partial sequences or clones were not collected for analysis. Phylogeny was determined by the minimum evolution method and given in the form of a complete phylogenetic tree. A data set of all 59 molecules was also given. MEGA6 bio software was used to align beta-satellite sequences. Sequences were aligned in the muscle alignment section by eliminating gaps between nucleotides, and a tree was constructed by the minimum evolution method for differences [11]. Gamma Parameter for Site Rates was also performed in the MEGA6 Program by the maximum likelihood method using the GTR (General time reversible) model [12]. Substitution Matrix Estimation was also prepared in the form of a table by using the maximum likelihood method with the shape parameter of the gamma distribution. The Neutrality Test of Tajima was also conducted to check nucleotide diversity and the number of segregating sites [12]. The sequence demarcation tool software (SDT) was used for matrix construction of beta-satellites [13]. Pairwise identity was also determined by using this Program. The SDT plot was given [14]. Recombination among chilli leaf curl beta-satellite sequences was checked by the RDP program (version 4). Different indicators were used to check recombination, including BootScan, SiScan, Chimaera, MaxChi, RDP, GENCONV, and 3Seq [15].

The p-value was set at 0.05, and the test was repeated three times, with a large step size (20+ nucleotides) used for confirmation. The RDP data set was also constructed to highlight the recombination and major and minor parents of a particular sequence.

## RESULTS

A total of 59 beta-satellite sequences associated with *ChiLCD* were retrieved from the database of NCBI. These sequences were reported from fifteen (15) different crops, including tomato, chilli, potato, weeds, mint, cotton, Cyamopsis, squash, okra, cannabis, mung bean, mash bean, pepper, and *Glycin* max (gene bank file). Twenty-two (22) betasatellite molecules were found in chilli crop, sixteen (16) from pepper, and three (3) from tomato crop, five (5) from cannabis, eleven (11) from okra, one (1) from mung bean, and one (1) from mash bean. Beta-satellite molecules were also found in *Glycin* max, weeds, and insects. A total of 59 satellite molecule sequences were aligned in MUSCLE alignment format, and a tree was constructed by the minimum evolution method. Bootstrap value was 90% with the Standard Non-parametric bootstrap method. Eighteen (18) clades were present in the phylogenetic tree. *ChiLCB* was found to infect chilli crop in 5 clades and pepper crop in 2 clades. An ideal tree with a total branch length of 0.96010526 was produced by estimating the evolutionary history using the Minimum Evolution (ME) approach. Using the Maximum Composite Likelihood approach, evolutionary distances were computed as base substitutions per site. At search level 1, the Close-Neighbor-Interchange (CNI) method was used to refine the ME tree. The Neighbor-Joining approach was used to build the first tree. 59 nucleotide sequences covering noncoding regions and codon positions 1–3 were included in the analysis. A final dataset of 1,193 positions was obtained by removing any positions with gaps or missing data. MEGA6 software was used for all evolutionary analyses. Beta satellite having accession number OQ076340 isolated from

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chilli crop appears at the bottom of the phylogenetic tree. The phylogeny tree is showing that chilli and pepper are the major hosts due to 38 isolated sequences. Other molecules are also infecting medicinal plants and vegetable plants (Figure 1).

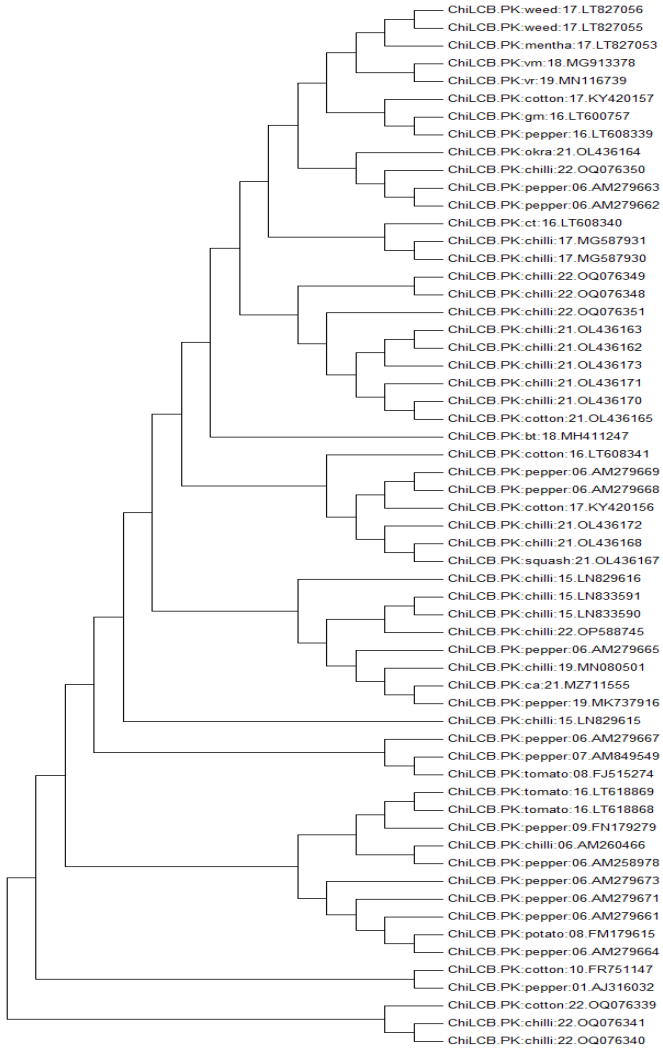


**Figure 1:** Dendrogramic Tree of *Chilli Leaf Curl* Beta-Satellite Isolated from Different Crops

The likelihood of a base (row) being substituted with another base (column) is represented by each entry (r). This model was used to estimate the substitution rates and patterns. Whereas transversional substitution rates are italicized, transitional substitution rates are in bold. These were kept under a single table. When assessing instantaneous r, relative values should be taken into account. In order to keep things simple, the nucleotide frequencies are as follows: T/U = 25.56%, A = 35.74%, G = 19.25%, and C = 19.45%. A hierarchy of trees was generated by software for the purpose of predicting ML values. For this calculation, the greatest Log likelihood was -8653.552. There were 59 nucleotide sequences in the analysis. Codon positions 1st+2nd+3rd+Noncoding were included. Positions with gaps and incomplete data were all removed. The complete database contained 1193 locations altogether. Evolutionary studies were carried out in MEGA6. Negative result of 'D' indicates that sequences undergo recent mutations with low-frequency polymorphism (Table 1).

**Table 1:** Substitution of *ChiLCB* among Sequences

| Variables | A | T/U | C | G |
|---|---|---|---|---|
| A | — | 6.52 | 4.96 | 7.10 |
| T/U | 9.12 | — | 12.40 | 4.91 |
| C | 9.12 | 16.30 | — | 4.91 |
| G | 13.18 | 6.52 | 4.96 | — |

The analysis includes 59 nucleotide sequences from 1193 sites, spanning all codon and noncoding areas (excluding incomplete data). MEGA6 was used to carry out evolutionary analysis. Negative result of 'D' indicates that sequences undergo recent mutations with low-frequency polymorphism. This has happened due to two main reasons: one is population expansion, in which the population expands with low mutation without losing genetic drift. The other reason is purifying selection, in which the virus undergoes a clean-out phase to remove harmful mutations (Table 2).

**Table 2:** Results from Tajima's Neutrality Test

| *m* | *S* | $p_s$ | $\Theta$ | $\pi$ | *D* |
|---|---|---|---|---|---|
| 59 | 569 | 0.476949 | 0.102652 | 0.072049 | -1.066996 |

*Abbreviations: D* is the Tajima test statistic, *n* = total number of sites, $\pi$ = nucleotide diversity, *S* = Number of segregating sites, *m* = number of sequences, $\Theta = p_s/a_1$, and $p_s = S/n$

*ChiLCB* isolated from chilli crop having accession number LN829615 was showing 100% resemblance to *ChiLCB* infecting cotton crop. Transmission would happen through trade between the materials of these two crops. Blue colour indicated the least resemblance of sequences among each other. The extreme red colour was showing 100% resemblance. The value of scale was kept between 94-82 to check homology and resemblance with each other (Figure 2).

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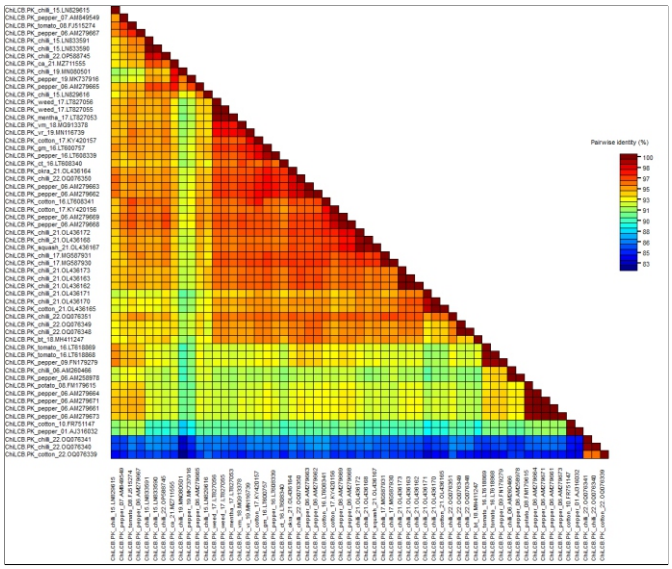

**Figure 2:** Matrix of *ChiLCB* Infecting Different Crops

The plot of the SDT analysis shows the percentage of nucleotide diversity. Maximum pairwise identity was calculated at 96 when the proportion of pairwise reached the maximum value of 0.16 (Figure 3).

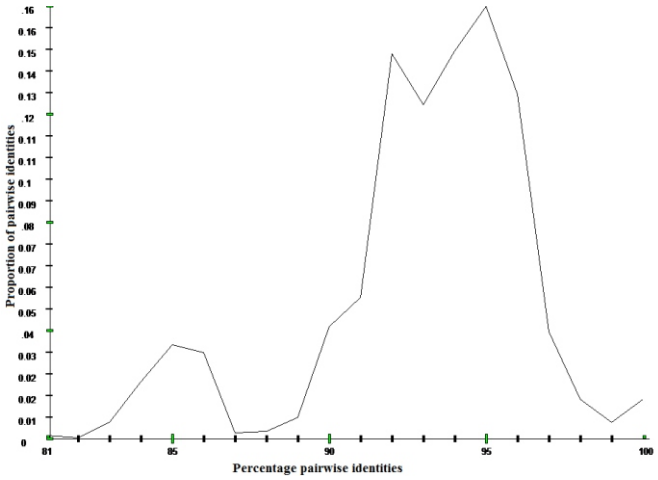


**Figure 3:** Percentage Pairwise Identities

All 59 sequences of beta-satellites were tested against the RDP Program to check for recombination in sequences. Thirty-eight (38) sequences were found to be unique and do not show any recombination. Recombination would happen due to the exchange or mixing of one viral DNA with another viral DNA. Beta-satellite sequence isolated from chilli crop base of the phylogenetic tree was found recombined. The sequence isolated from Pakistan from the tomato was the major parent, and the minor parent was okra (RDP test). A complete overview of the RDP of selected tests was given. Results were confirmed three times by using different methods. Parents of some sequences were unknown. Parents of some sequences were found in weed crops (Table 3).

**Table 3:** Complete Overview of RDP Program

| Event no | Found in | Recombinant | Major Parent | Minor Parent | Detection Methods | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | R | G | B | M | C | S | T |
| 1 | 1 | *ChiLCB*.PK: pepper | *ChiLCB*.PK:ca | Unknown | - | + | + | + | - | + | + |
| 2 | 1 | *ChiLCB*.PK:vr | *ChiLCB*.PK: cotton | *ChiLCB*.PK:vm | - | - | + | + | - | + | + |
| 3 | 7 | *ChiLCB*.PK: chilli | Unknown | *ChiLCB*.PK: chilli | + | + | + | + | + | + | + |
| 4 | 3 | ChiLCB.PK: pepper | *ChiLCB*.PK: chilli | *ChiLCB*.PK: potato | - | + | + | + | - | + | - |
| 5 | 1 | *ChiLCB*.PK: chilli | *ChiLCB*.PK: tomato | *ChiLCB*.PK:okra | - | - | - | + | - | + | + |
| 6 | 2 | *ChiLCB*.PK: chilli | *ChiLCB*.PK: chilli | *ChiLCB*.PK: chilli | + | + | + | + | + | + | + |
| 7 | 1 | *ChiLCB*.PK:bt | *ChiLCB*.PK: okra | Unknown | + | + | + | - | - | + | + |
| 8 | 4 | *ChiLCB*.PK:gm | *ChiLCB*.PK:ct | Unknown | - | - | - | + | + | - | + |
| 9 | 6 | *ChiLCB*.PK: pepper | *ChiLCB*.PK: gm | *ChiLCB*.PK: tomato | - | - | - | - | - | + | - |
| 10 | 2 | *ChiLCB*.PK: pepper | Unknown | *ChiLCB*.PK: chilli | - | + | + | + | - | + | - |
| 11 | 3 | *ChiLCB*.PK: tomato | Unknown | *ChiLCB*.PK: chilli | - | - | - | + | + | + | + |
| 12 | 6 | *ChiLCB*.PK:bt | *ChiLCB*.PK: chilli | *ChiLCB*.PK: chilli | - | - | - | - | - | + | - |
| 13 | 3 | *ChiLCB*.PK: cotton | Unknown | *ChiLCB*.PK: chilli | - | + | + | - | - | - | - |
| 14 | 7 | *ChiLCB*.PK: chilli | Unknown | *ChiLCB*.PK: pepper | + | + | - | + | - | + | + |
| 15 | 1 | *ChiLCB*.PK: chilli | Unknown | *ChiLCB*.PK: pepper | - | - | - | + | + | + | + |
| 16 | 4 | *ChiLCB*.PK: chilli | *ChiLCB*.PK: chilli | Unknown | - | - | - | - | - | + | - |
| 17 | 6 | *ChiLCB*.PK: chilli | *ChiLCB*.PK: chilli | *ChiLCB*.PK: pepper | - | - | - | + | + | - | + |
| 18 | 1 | *ChiLCB*.PK: | *ChiLCB*.PK: cotton | *ChiLCB*.PK: pepper | - | - | - | - | - | + | - |
| 19 | 1 | *ChiLCB*.PK: chilli | *ChiLCB*.PK: chilli | Unknown | - | - | - | - | - | - | + |
| 20 | 2 | *ChiLCB*.PK:vr | *ChiLCB*.PK: menthol | *ChiLCB*.PK: cotton | - | - | - | - | - | + | - |
| 21 | 2 | *ChiLCB*.PK: cotton | *ChiLCB*.PK:vr | *ChiLCB*.PK: cotton | - | - | - | - | - | + | - |

T= 3seq, S= SiScan, C= Chimaera, M= MAXchi, B= Boot-Scan, G= GENCOV, R= RDP

Twenty-one (21) sequences were found recombined with a frequency of 35.6%. In *begomoviruses*, this value indicates that

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recombination is a primary source of genetic diversity. Major parents indicate a large proportion of fragments contributing towards viral replication and integrity. Minor parents make a portion of some important genes, like coat protein, which makes the virus more virulent and makes it adaptable to new species (Figure 4).

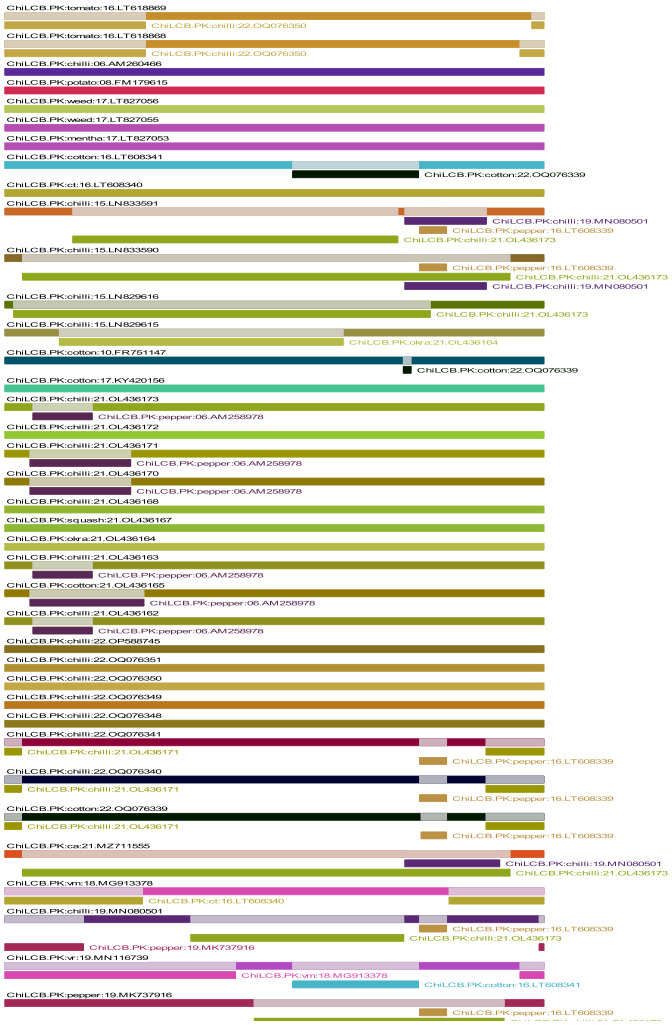


**Figure 4:** RDP Test Showing Major and Minor Parents of the Sequences

## DISCUSSION

Gradual change in DNA is a continuous process in nature. It helps viruses to evolve with the passage of time and adapt to new crop species [16, 18]. The history of the evolution of viruses is much older than we think. However, their evolution records are not available properly; therefore, scientists are relying on present viruses to determine their history, their ancestors, and the mechanisms by which they evolve, what factors are affecting their evolution and emergence, etc. Through mutation and recombination, *geminiviruses* evolved gradually, and many natural factors, including chemical, biological and physical were also assisting this mechanism. According to some studies, plant viruses were present in nature about 450 million years ago. With the passage of time, these viruses have been discovered with different features and properties. Some *begomoviruses* isolated from India and Vietnam showed unique characteristics of old viruses. So, it can be said that these are descendants of old viruses. Phylogenetic studies have shown that old world *begomoviruses* (OW) are forefathers of new world *begomoviruses* (NW). Besides a gradual change in DNA, sudden changes in DNA also play an important role in virus evolution and the arrival of new species. In recent times, in Pakistan, a new strain of cotton leaf curl virus proved very devastating to the cotton crop. Experiments have been conducted on Tobacco etch virus (*TEV*) to check its effectiveness when it evolves. These experiments tell the virulence of *TEV* after evolution. Experiments also reveal that there is an increase in biotypes after evolution [19]. Artificial mutations were induced in *TEV* and tested against different host plants [20]. Results have revealed that the virulence of *TEV* increases as the number of mutations increases, but adaptability to new crops and environments decreases. A previous study has shown that a mutated strain of *TEV* was virulent against pepper but not against tobacco [21]. When this infected plant sap is inoculated into tobacco plants, the tobacco plants show equal symptoms. This phenomenon is known as a pleiotropic effect [22]. Studies have shown that recombination frequently occurs in the *Bromoviridae* family. RNA viruses in the *Bromoviridae* family evolve more quickly than DNA viruses [23, 24]. In the cucumber mosaic virus (*CMV*), recombination occurs at a faster rate. Different strains with recombination showed different degrees of virulence [25]. Greater mutations do not necessarily mean a higher degree of virulence, but if virulence increases due to mutation, adaptability to new plants and environments decreases [26]. Gene bank analysis of Chilli ring spot virus (*ChiRSV*) revealed that it has been isolated from three different countries so far. Phylogenetic analysis of *ChiRSV* revealed that the sequence with accession number KT633930, isolated in China, serves as the basis and supports further evolution and transmission of this virus [27]. Our phylogenetic analysis suggested that *OLCuA* isolated from Oman was an ancestral sequence and provided a platform for further evolution and distribution [28]. *Begomoviruses* are the most notorious viruses regarding replication, evolution, and transmission. Their plasmid-like replication makes them more virulent to infect more crop species. Pepper leaf curl Lahore virus (*PepLCLV*) is showing great diversity in chilli and pepper crops across the subcontinent. SDT analysis of *PepLCLV* indicated that some sequences showing 100% homology with each other, while they are isolated from different countries [29]. In this article, our SDT analysis is also showing 100% similarity in some

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sequences. *Geminiviruses* are thought to be very diverse among plant viruses. Chickpea chlorotic dwarf virus (*CpCDV*), a mastrevirus belonging to the *Geminiviridae* family, infects diverse plant species. Data analysis revealed that it not only infects the *Fabaceae* family but also the *Malvaceae* family [30].

Despite the knowledge acquired, this study has a number of drawbacks. First, because data from some places is underrepresented in comparison to others, the dependence on sequences found in gene banks introduces a geographical bias. This could distort evolutionary interpretations of ancestral origins, like those proposed for *OLCuA* and *ChiRSV*. Second, there is no wet-lab validation to verify the biological effects of certain mutations or recombination events on host-pathogen interactions, and the evolutionary inferences derived from sequence homology and phylogenetic analysis are solely computational. Lastly, despite the limitations of molecular clock analysis, it is challenging to definitively recreate the timeline of viral development due to the fragmented nature of historical viral data. To address these limitations and advance the understanding of viral evolution, future research should prioritize the following areas, which include Comprehensive Scrutiny to create a more comprehensive worldwide picture of viral diversity. Functional authentication to confirm the biological importance of detected recombination events and mutations. environmental factor analysis to better predict emergence patterns, future research should look into the particular biological and environmental stressors (such as vector population dynamics and climate change variables) that cause the high recombination rates seen in *Bromoviridae* and *Geminiviridae.*

## CONCLUSIONS

Satellite molecules were downloaded from the database. Different evolutionary tests were conducted on them. A phylogenetic tree and SDT matrix were created using different software. Recombination was detected by using the RDP Program. Eighteen (18) clades were present; five (5 clades were found only in the chilli crop. In the SDT plot, the resemblance value was kept between 82 and 94, and the sequences showed a maximum proportion of homology at 0.02. In the RDP test, 21 sequences were found to be recombined with major and minor parents. This means that viruses also get some proportion of their genomes from other viruses. Seven different types of selected tests were applied in the RDP program.

## Authors' Contribution

Conceptualization: MA
Methodology: MA, UB, QI
Formal analysis: UB, MTM
Writing and Drafting: UB
Review and Editing: MA, UB, MTM, QI, MZ

All authors approved the final manuscript and take responsibility for the integrity of the work.

## Conflicts of Interest

All the authors declare no conflict of interest.

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**Original Article**

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# Synthesis and Biological Activities of Cactus-Mediated Silver Nanoparticles

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## ABSTRACT

The field of nanotechnology is expanding swiftly along with the diversified antioxidant and antibacterial applications of silver nanoparticles. **Objectives:** To articulate AgNPs using pigmented extract from two cactus varieties i.e., Rattail cactus (*Disocactus flagelliformis*) and Moon Cactus (*Gymnocalycium mihanovichii*). **Methods:** Using a green synthesis methodology, silver nitrate solution and both cactus extracts were mixed and incubated, followed by a color variation detection. UV-visible, and FTIR analysis were performed to validate the synthesis of silver nanoparticles. Antioxidant activity was assessed using DPPH assay, whereas well diffusion methods evaluated the antibacterial ability of cactus nanoparticles. **Results:** Blending of aceto-water extract of cactus plant with silver nitrate resulted a discrete color change from yellow to blackish brown. UV–Visible spectroscopy demonstrated surface plasmon peaks at 250 nm as well as at 400-450 nm. Fourier Transform Infrared (FTIR) spectroscopy identified interactions in nanoparticle samples due to specific functional groups in cactus pigmented extract. Antioxidant capacity was appraised via DPPH assay at 500 ppm and 1000 ppm, enlightening enhanced radical scavenging (31-48%) with increased concentration, exclusively at 1000 ppm. Antibacterial efficacy was verified by agar well diffusion against *Escherichia coli* and *Staphylococcus aureus*. It revealed distinct zones of inhibition, with greater activity against *E. coli* (17mm zones of inhibition) versus *Staphylococcus aureus*. **Conclusions:** The results of our study place Moon and Rattail Cactus pigment extracts as active green assets for AgNP fabrication, emphasizing their value in biomedical advances.

## INTRODUCTION

Microscopic particles ranging from 1 to 100 nanometers (nm) in size are called nanoparticles. The main factors that determine the properties of nanoparticles (NPs) are their sizes and shapes. It is important to synthesize nanoparticles with an appropriate size, structure, monodispersed, and morphology to achieve the particular uses [1]. In nanotechnology, green technology has come forward that is environmentally friendly and can be utilized to reliably produce nanomaterials and nanoparticles from non-toxic green plants. The presence plays a crucial role in plant-aided reduction of metal ions. Plants' flavonoids, terpenoids, and alkaloids are directly involved in the reduction and thus design of silver nanoparticles. These phytochemicals act as reducing agents by reducing metal ions into their metallic form and also serve as stabilizing and capping agents, preventing the aggregation of nanoparticles [2]. Various researchers have explored the potential of medicinal plants for synthesizing nanoparticles with unique properties [3]. However, there still exists the need for exploring more plants for the cost-effective synthesis of nanoparticles. Moon cactus (*Gymnocalycium mihanovichii*) is primarily a decorative plant; however, its fever-reducing property, anti-inflammatory properties, and antioxidant activities (due to its betalain pigments) have been reported [4, 5]. Another variety of cactus, i.e., Rattail cactus (*Disocactus*

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*flagelliformis*), is another member of the family Cactaceae, which has been depicted to have anticancer and anti-inflammatory properties that urged us to explore its potential for nanoparticles synthesis [6]. In recent years, silver nanoparticles (AgNPs) have emerged as the most popular nanostructures with promising properties suitable for various biological applications. They exhibit broad-spectrum antimicrobial activity and thus can be employed for anticancer and antimicrobial therapy, wound repair, and bone healing. The role of silver nanoparticles as biosensors is also well documented [7, 8]. Silver particles are well known as drug carriers, enhancing bioavailability and targeting specific cells or tissues [9]. A variety of Cactus species (*Euphorbia cactus, Ethiopian cactus,* and *Opuntia ficus-indica* Cactus plant) have shown their silver nanoparticle synthesis potential.

However, two important cactus varieties, like moon and rattail cactus, were seen to have specific betalain pigments with antioxidant power, and these species have not been explored yet for their reducing potential or nanoparticle synthesis. To fill this gap, this research aimed at exploring and comparing the potential of, i.e., moon and rattail cactus, to synthesize silver nanoparticles, to characterize the silver nanoparticles via UV-Visible spectroscopy and FTIR analysis. Moreover, to study the antimicrobial and antioxidant activities of formed cactus mediated silver nanoparticles.

## METHODS

This experimental study was performed at the Institute of Biochemistry, University of Sindh, Jamshoro, during August-December 2025. Red tail cactus flowers and Moon Cactus colored parts were collected from a local nursery and recognized by the Department of Plant Sciences, University of Sindh. Plants were rinsed with distilled water and ground using a mortar and pestle to obtain the paste. This mixture was soaked in acetone and water in a 1:1 ratio to fully immerse the material, and the bottle was stored in a dark place for 24 hours. After 24 hours, the mixture was centrifuged to obtain a clear plant extract. 1 mM silver nitrate solution was prepared and used as the metal ion source. For the synthesis reaction, the plant extract was slowly introduced into the silver nitrate solution in a 1:9 ratio under continuous stirring. The reaction mixture was kept at room temperature in dark conditions [10]. The reduction of silver ions was indicated by a gradual color transition of the reaction mixture from transparent to brown, confirming the synthesis of silver nanoparticles. The mixture was allowed to react for 24 hours to achieve maximum nanoparticle formation. The synthesized nanoparticles were separated by centrifugation at 10,000 rpm for 15 minutes. The collected pellets were washed repeatedly with deionized water to eliminate residual plant compounds. The purified silver nanoparticles were re-suspended in deionized water and stored at 4 °C in light-protected containers for subsequent analysis [11]. For UV-visible analysis, three dilutions of each extract were prepared: 1 mL, 2 mL, and 4 mL. These dilutions were scanned across the UV-Visible range of 400-800 nm. Absorbance values and λmax were recorded for each scan and graphs were generated using Origin 2020 software. FTIR analysis included the direct placement of nanoparticle powder on a PerkinElmer Spectrophotometer over the wavenumber range of 4000–400 $cm^{-1}$. Antioxidant potential of synthesized nanoparticles was evaluated via DPPH Assay. For this, fresh 0.1mM DPPH dissolved in methanol, and 1ml of this prepared DPPH was allowed to mix with 1ml of nanoparticles suspension (500 and 1000 ppm). The test tubes were incubated in the dark for 30 minutes at 37°C, and then absorbance was recorded at 517 nm along with three replicates [3]. All antioxidant and antibacterial assays were performed in triplicate technical replicates using three independently synthesized nanoparticle batches (biological replicates) to ensure reproducibility and reliability of results. The synthesis of silver nanoparticles using each cactus extract was performed independently three times (n=3 biological replicates). For each biological replicate, all subsequent assays (UV-Vis, FTIR, DPPH, antibacterial) were conducted in technical triplicate. Results from the three independent syntheses were pooled for statistical analysis. Cactus nanoparticles in 500ppm and 1000ppm concentration were dissolved in methanol for antibacterial analysis. Firstly, bacterial cultures were developed on nutrient agar media for 24hrs at 37°C. *E. coli* and *S. aureus* bacterial suspensions were swabbed evenly on the entire autoclaved agar surface. Using the well diffusion method, equal-diameter wells were formed in the agar using a sterile corn borer. About 50µl of cactus silver nanoparticles was mixed in each well, and this was followed by incubating the plates at 37ºC for 24 hours. This was repeated thrice for the same sample to achieve accuracy [12]. The next day, Zones of inhibition were measured to assess antibacterial activity. . To avoid observer bias, all measurements (zone of inhibition diameters, UV-Visible absorbance readings, DPPH absorbance values) were performed by a researcher blinded to the sample identities. Samples coding was done by independent person and codes were not revealed.

All experiments were performed with three independent nanoparticle syntheses (biological replicates, n=3), each measured in technical triplicate. Results are presented as mean ± standard deviation (SD). Blinding was applied during all measurements to avoid observer bias. For DPPH data, one-way ANOVA and regression were used. For antibacterial data, unpaired two-tailed t-tests compared zones of inhibition between bacterial species and between

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concentrations. A p-value<0.005 was considered statistically significant. 95% confidence intervals (CIs) are reported. Statistical analyses were performed using GraphPad Prism 9.0.

## RESULTS

Cactus extract and metal salt were mixed and incubated, then a color change was observed, confirming the synthesis of nanoparticles. Absorbance intensity was seen to be increased with volume, highest for 4 mL and lowest for 1mL. However, the 4 ml sample showed much noise, which needs to be corrected by diluting the sample (Figure 1).

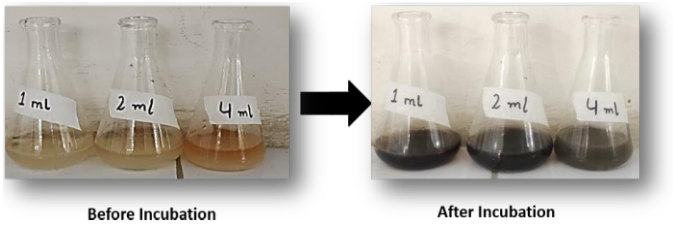


**Figure 1:** Color Change Showing the Synthesis of Nanoparticles

The UV-Visible spectroscopy analysis confirms the successful green synthesis of silver nanoparticles (AgNPs) using moon cactus (a) and rat-tail cactus plant extract (b), as evidenced by the characteristic surface plasmon resonance (SPR) peaks in 250-300 nm, as well as peaks were also seen at 350 nm and 400-450 nm, particularly with the use of 4 ml cactus extract (Figure 2).

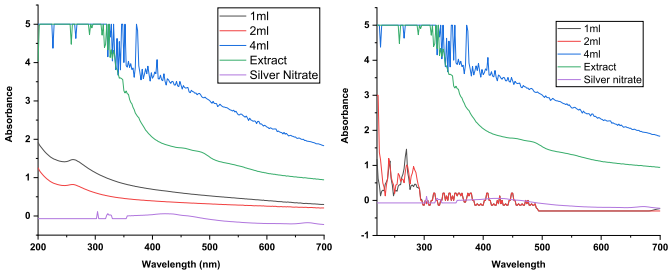


**Figure 2:** UV-Visible Absorbance Spectra of Silver Nanoparticles (AgNPs) Biosynthesized Using Moon Cactus Extract (a) and Rat-tail Cactus Extract (b)

FTIR analysis demonstrated a broad band at 3000–3500 $cm^{-1}$, suggestive of O-H stretching vibrations, which indicate hydroxyl-rich cactus biomolecules' contribution to nanoparticle synthesis/ stabilization. Other peaks at 1000 $cm^{-1}$ and 1500–1800 $cm^{-1}$ were seen to be involved in nanoparticle synthesis (Figure 3).

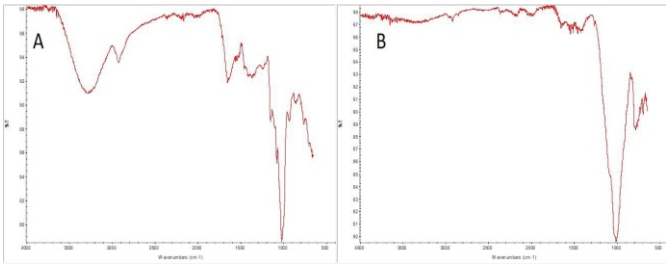


**Figure 3:** FTIR Spectra of (A) Rattail Cactus Nanoparticles and (B) Moon Cactus Nanoparticles

In an antibacterial assay, the presence of well-defined clear zones indicates suppression of bacterial growth in the surrounding area (Figure 4).

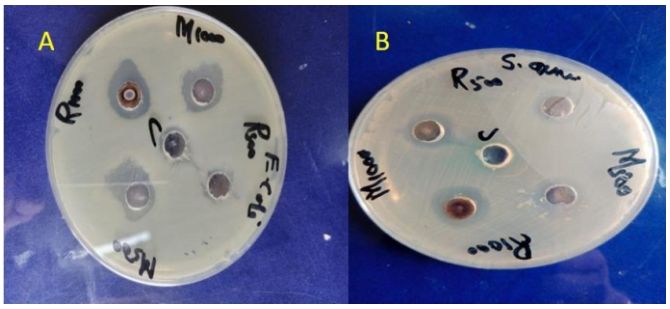


**Figure 4:** Zones of Inhibition of Moon and Rattail Cactus Silver Nanoparticles Against (a) *E. coli* and (b) *S. aureus*

In contrast, the negative control did not show any zone of inhibition, confirming that the observed antibacterial activity was solely due to the nanoparticle treatment. The overall efficacy of the current AgNPs against Gram-negative *E. Coli* was visually confirmed as potent, i.e., 17 mm zone of inhibition. Rattail cactus was found to inhibit S. aureus with the highest zone of inhibition (i.e., 16 mm) at 1000ppm concentration. Negative control (Distilled water) showed negligible zones, while the highest zones of inhibition were shown by ciprofloxacin (positive control) (Table 1).

**Table 1:** Zones of Inhibition (mm)

| Samples | Zones of Inhibition (mm) | |
|---|---|---|
| | *E. Coli* | *S. aureus* |
| M.C. 500 ppm | 13 | 12 |
| M.C 1000 ppm | 17 | 14 |
| R.C 500 ppm | 11 | 09 |
| R.C 1000 ppm | 17 | 16 |
| Distilled Water (-ve control) | 00 | 00 |
| Ciprofloxacin | 21 | 19 |

The antibacterial assay revealed concentration-dependent activity (Table 1). Moon cactus AgNPs at 1000 ppm showed significantly greater inhibition against *E. coli* (17.0 ± 0.58 mm) compared to *S. aureus* (14.0 ± 0.58 mm, p = 0.01, 95% CI: 1.48–4.52), confirming statistically superior activity against the Gram-negative bacterium. Similarly, Rattail cactus AgNPs at 500 ppm showed a significant difference (*E. coli*: 11.0 ± 0.58 mm vs *S. aureus*: 9.0 ± 0.58

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mm, p = 0.03, 95% CI: 0.48–3.52). Positive control (ciprofloxacin) produced expected inhibition zones (21.0 ± 0.58 mm for *E. coli*, 19.0 ± 0.58 mm for *S. aureus*), validating the assay (Table 2).

**Table 2:** Zones of Inhibition against *E. coli* and *S. aureus* (n=3)

| Samples | *E. coli* (Mean ± SD) | *S. aureus* (Mean ± SD) | p-value* | 95% CI |
|---|---|---|---|---|
| Moon Cactus (500 ppm) | 13.0 ± 0.58 | 12.0 ± 0.58 | 0.080 | (-0.52, 2.52) |
| Moon Cactus (1000 ppm) | 17.0 ± 0.58 | 14.0 ± 0.58 | 0.010 | (1.48, 4.52) |
| Rattail Cactus (500 ppm) | 11.0 ± 0.58 | 9.0 ± 0.58 | 0.030 | (0.48, 3.52) |
| Rattail Cactus (1000 ppm) | 17.0 ± 0.58 | 16.0 ± 0.58 | 0.080 | (-0.52, 2.52) |
| Distilled Water (-ve) | 0.0 ± 0.00 | 0.0 ± 0.00 | – | – |
| Ciprofloxacin (+ve) | 21.0 ± 0.58 | 19.0 ± 0.58 | 0.020 | (0.48, 3.52) |

*Unpaired t-test

In the DPPH Assay, as we added the DPPH Solution to the sample solution, a clear change in color was observed from purple to yellowish brown. A color change showing transition towards a pale-yellow shows good DPPH scavenging (Figure 5).

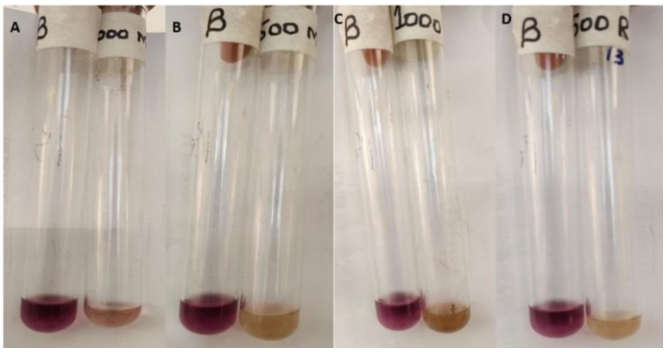

**Figure 5:** Color Change Showing DPPH Scavenging by (a) Moon Cactus NPs1000ppm, (b) Moon Cactus NPs 500ppm, (c) Rattail Cactus NPs 1000ppm, (d) Rattail Cactus NPs 500ppm

When calculated by the mentioned formula, DPPH percent inhibition by cactus nanoparticles was found to be increased in a concentration-dependent manner, i.e., 48% for 1000 ppm and 31% for 500 ppm. The $IC_{50}$ value of Moon cactus AgNPs (1052 ppm) was lower than that of Rattail cactus AgNPs (1587 ppm), indicating the superior antioxidant potency of Moon cactus-derived nanoparticles. However, both values are considerably higher than ascorbic acid (10 ppm), suggesting that further optimization of synthesis conditions (e.g., reducing aggregation, improving capping efficiency) could enhance radical scavenging activity (Table 3).

**Table 3:** DPPH Percent Scavenging of Silver Nanoparticles

| Samples | Scavenging Activity (%) | $IC_{50}$ (ppm) | 95% CI ($IC_{50}$) | p-value |
|---|---|---|---|---|
| Moon Cactus (1000 ppm) | 47.9 ± 0.60 | 1052 | (987–1123) | <0.001 |
| Moon Cactus (500 ppm) | 31.46 ± 0.06 | 1052 | (987–1123) | – |
| Rattail Cactus (1000 ppm) | 31.71 ± 0.01 | 1587 | (1492–1689) | – |
| Rattail Cactus (500 ppm) | 11.17 ± 0.005 | 1587 | (1492–1689) | <0.001 |
| Blank (DPPH Only) | 0.07 ± 0.00 | – | – | – |
| Ascorbic Acid (Positive Control) | – | 10 | (8.5–11.8) | <0.001 |

The high $R^2$ values (>0.94) and significant p-values (<0.005) confirm a strong, statistically significant positive linear relationship between cactus extract volume and absorbance intensity, validating the concentration-dependent increase in nanoparticle yield (Table 4).

**Table 4:** Linear Regression Parameters for Absorbance Intensity Versus Cactus Extract Volume

| Parameters | Moon Cactus AgNPs | Rat-tail Cactus AgNPs |
|---|---|---|
| $R^2$ | 0.964 | 0.941 |
| p-value (Slope ≠ 0) | 0.008 | 0.011 |
| 95% CI for Slope | (0.048, 0.126) | (0.036, 0.122) |

## DISCUSSION

A visible color change as well as UV visible analysis confirmed that the nanoparticle samples (with sample volumes of 1 and 2 ml) exhibited a sharp SPR peak around 250-300 nm, different from the usual SPR at 400-450 nm. Although such absorption peaks are not considered as primary SPR for silver nanoparticles synthesis, when 250 nm is present with a 450 nm peak, then it may indicate the formation of some silver nanoclusters. It may suggest that phyto-organic compounds involved in silver reduction are still present in the current sample [13]. This may be due to residual phytochemicals of Cactus in nanoparticles samples, clumping or agglomeration of synthesized AgNPs, as when nanoparticles clump, then the SPR peak may disappear when they are oxidized to silver. It may be corrected by using strategies such as dilution of the UV-visible sample, increasing reaction incubation time [14], and characterizing the nanoparticles via SEM and DLS. Absorbance intensity was increased in the case of 4 ml, which reflects more silver reduction and nanoparticle yield from phytochemicals in the cactus extract [15, 16]. The 4 ml sample depicts sharp peaks around 380 nm and 420 nm, but due to noise, it may not reflect the actual SPR band, so it needs to be diluted and repeated again. These results validate that the rat-tail cactus acts as an efficient bio-reductant and stabilizer for eco-friendly AgNP production. FTIR analysis showed sharp peaks at ~1000 $cm^{-1}$, which is suggestive of the C-O stretching vibration of either alcohol or phenolic compounds from cactus extract. It indicates that phenols or flavonoids in cactus extract have actively participated in reducing silver ions as well as capping them. Sharp peaks in the 1500–1560$cm^{-1}$ range were observed in the rattail cactus nanoparticles sample, which shows amide II band or aromatic vibrations often came from flavonoids [17]. The synthesized nanoparticles displayed visible inhibitory effects against both Gram-negative and Gram-positive bacteria, i.e., *E. Coli* and *S. aureus*,

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respectively. The Antibacterial efficacy of plant-mediated nanoparticles is possible due to their large surface area and nanosize, which helps them to strongly interact with the bacterial cell membrane [18]. Reported results of such membrane interactions include membrane damage, enhanced permeability, and intracellular contents leakage, eventually causing cell death. Additionally, induction of reactive oxygen species (ROS) is also reported, which leads to oxidative stress and can cause damage to vital cellular constituents such as proteins or DNA. The higher susceptibility of *Escherichia coli* compared to *Staphylococcus aureus* may be due to the presence of an outer membrane in its structure and cell wall differences [19]. DPPH analysis suggested that moon cactus-derived silver nanoparticles were more efficient in scavenging DPPH free radicals (i.e., 48%) as compared to rattail cactus at 1000 ppm concentration. This difference in antioxidant potential may be due to differences in pigment composition and varied phytochemicals that possess free radical scavenging ability [20, 21]. Current results are different from the already reported Euphorbia Cactus nanoparticles' DPPH potential of 96.12% at a concentration of 80 µg/ml [22]. It may be improved after correcting the problems of aggregation and correcting UV-visible surface plasmon readings.

Some limitations of this study include a lack of funding, due to which advanced characterization techniques could not be performed. Moreover, IC50 values of cactus nanoparticles need to be calculated via GraphPad Prism. Future studies should focus on characterizing these nanoparticles via SEM, XRD, and zeta potential to know their specific properties. Stability studies and in vivo assays would further help us to determine the toxicity of cactus nanoparticles before their use in biomedical applications.

## CONCLUSION

Moon cactus and rattail cactus were explored for their potential to synthesize silver nanoparticles. Blending of both cactus extract with silver salt resulted in a visible color change from yellow to blackish brown. Specific SPR peaks were absent while using 1 ml and 2 ml cactus extract, whereas 4 ml cactus extract was shown to exhibit peaks around 420 nm, although its noise needs to be corrected by using diluted samples. FTIR analysis depicted phenols and flavonoids of Cactus as major reducers and capping partners. Fabricated silver nanoparticles showed 31-48% DPPH percent scavenging and a pronounced zone of inhibition (17mm) against E. coli at 1000 ppm concentration. Therefore, these nanoparticles need to be further characterized and explored for their use as strong antimicrobials and robust green antioxidants.

## Authors' Contribution

Conceptualization: BK
Methodology: SN, AAK
Formal analysis: AAK, AR
Writing and Drafting: BK, AAK, MW, AR
Review and Editing: BK, SN, AAK, MW, AR

All authors approved the final manuscript and take responsibility for the integrity of the work

## Conflicts of Interest

All the authors declare no conflict of interest.

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