INVESTIGATING THE RELATIONSHIP BETWEEN *VEGF* GENE POLYMORPHISM (RS699947) AND DENGUE SUSCEPTIBILITY AND *IN-SILICO* STUDY OF ANTI DENGUE BIOACTIVE COMPOUNDS

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Abstract. An increased risk of developing severe dengue has been linked to the *VEGF* gene. Angiogenesis, which is involved in developing and maintaining blood vessels, is controlled by the *VEGF* protein. In addition to influencing immunological responses, *VEGF* is a major contributing factor in the increased vascular permeability seen in severe dengue patients. Technology and transportation advancements have made it possible for dengue to travel quickly, and outbreaks of the disease are now occurring in several nations across several continents, putting billions of people's life in danger. This research aimed to perform mutational analysis of *VEGF* in dengue serotypes 1 and 2 patients and find potential antiviral inhibitors for dengue treatment. 144 samples were analyzed,72 cases and 72 controls. Mutational analysis was done by performing tetra arms PCR. Results showed no significant difference in allele frequency among patients and controls. 89% of samples and 96% of controls had Allele C. In comparison, 6% of samples and 3% of controls had Allele A ($p<0.0001$) showing heterozygous condition. Bioinformatic analysis was done to find potential antiviral phytochemicals that would be effective for the treatment of dengue. Upon bioinformatic analysis, artemisinin, sanguinarine, chelidonine, glycyrrhisoflavone, and taspine showed strong binding energies of -8.7 to -7.8 to NS-1viral protein. In conclusion, no genetic association existed between SNP and phenotypic traits of dengue. The extensive transmission and serious effects of dengue make it a serious worldwide health issue. To create efficient treatments and preventive measures to combat dengue, it is essential to understand the genetic and molecular features of the virus and host response.
Keywords: *angiogenesis, artemisinin, chelidonine, dengue, phenotypic*

Introduction

The globally prevalent dengue virus responsible for causing dengue, a formidable arboviral infection, is transmitted by *Aedes aegypti* mosquitoes (Ilyas et al., 2019). Its genome is membrane-enveloped ssRNA and is a member of the genus Flavivirus in the family Flaviviridae. Dengue exists in four antigenically related forms (DENV1-DENV4) (Murugesan and Manoharan, 2020). The disease spectrum ranges from mild febrile illnesses to severe conditions such as Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS), presenting a significant challenge for control and prevention strategies (Harapan et al., 2020). Globally, dengue is endemic in more than 100 countries, with 2.5 billion new infections reported annually (Khursheed et al., 2013) over the past 50 years, dengue cases have increased up to 30%, particularly in Asia (Ilyas et al., 2019).

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Genetic factors contribute to the variability in disease severity, with polymorphisms in the *VEGF* gene emerging as potential indicators of heightened risk for severe manifestations (Skrypnik et al., 2020). The *VEGF* gene has eight exons and seven introns and can be located on chromosome 6.p12-p21.A prominent *VEGF* polymorphism, the rs699947 polymorphism (*VEGF*-2578), is a mutation of C to A that may increase the risk of sickness with aberrant angiogenesis (Hussain et al., 2022). Recently, it has been proposed that the *VEGF* gene plays a role in the severity of dengue. As a regulator of angiogenesis and vasculogenesis, *VEGF* is essential for normal embryonic development and wound healing (Fiestas Solórzano et al., 2022).

This research initiative delves into the molecular intricacies of dengue infection, focusing on serotypes 1 and 2, and elucidates the interplay between genetic predisposition and disease outcomes. A comprehensive mutational analysis of the *VEGF* gene is undertaken, shedding light on its potential influence in shaping dengue susceptibility and severity. Notably, the *VEGF* protein's role in angiogenesis and immune modulation may contribute to the pathological vascular permeability observed in severe dengue cases (Hussain et al., 2023). Amidst the challenges posed by the rapid global spread of dengue, the pursuit of effective treatments and preventive measures remains paramount as its caused by four distinct serotypes (Wilder-Smith, 2022). Leveraging cutting-edge technologies, this study employs *in-silico* screening to identify potential antiviral compounds, offering a promising avenue for therapeutic intervention (Caraballo and King, 2014). The main objective of this research was mutational analysis of *VEGF* gene in Dengue serotypes 1 and 2 patients and to find plant antiviral inhibitors for the treatment for dengue as its clinically mismanaged and complicated due lack of antiviral drug.

Materials and methods

A total of 72 blood samples of patients with dengue were recruited in the study. Samples were collected from different areas of Lahore, Pakistan. The study was approved by the Departmental ethical committee of University of the Punjab. Patients confirmed with serotypes 1 and 2 were included in the study. A consent letter was signed by the study population. Blood samples of 72 healthy subjects were taken as controls. DNA extraction from whole blood was done by rapid DNA extraction method. Reported primers of rs699947 (Hussain et al., 2022) were used to amplify the *VEGF* gene (*Table 1*). 20 µl PCR reaction was prepared by utilizing 1 µl of DNA template, 15 µl PCR mixture, 0.5 µl each of inner and outer, i.e., forward and reverse, and 2 µl of nuclease-free water. The thermocycler was programmed with an annealing temperature of 67.3° for 30 s and 72° for 30 s as an extension time. The final extension was done for 5 minutes at 72°. The thermocycler took one and a half hours to complete its cycles. 1.3% agarose gel was prepared to visualize the DNA product of PCR. Product sizes were confirmed by the utilization of a 50 kb Ladder. Data was statistically analyzed using SNP stat software 1.52.0 for calculating chi-square and odds ratio. Hardy Weinberg equilibrium was used to calculate SNP variants and phenotypic traits and results are listed in *Table 2*.

In-silico screening of anti-NS1 dengue viral protein was done by retrieving the structure of NS-1 and antiviral phytochemicals from PDB and Dr. Duke. Impurities, Ligands, and water molecules were removed from the target NS-1 protein using PyMol (version 2.5.2). Data of phytochemicals used as ligands against target protein was collected from different articles and downloaded in SDF format from the PubChem database. Lipinski’s rule of five in SWISSADME checked the drug-likeliness of

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phytochemicals. Docking results were observed in PyMol (Naveed et al., 2023a, 2023b, 2024; Aziz et al., 2024).

Table 1. *Primers for VEGF (rs699947) gene (Hussain et al., 2022)*

SNP	Primers	Melting Temperature	Product size
rs699947	A allele GCCAGCTGTAGGCCAGACCCTGGTA	72°C	183 bp
	C allele CCAGTCAGTCTGATTATCCACCCAGACC		299 bp
	Controls CTAGTGCACGAATGATGGAAAGGGAGG AAGGCCCCATCCCATTCTTGCATATAGG		427 bp

Results

This study was conducted utilizing the lab and research resources of the IMMG, University of the Punjab-Lahore. In this research investigation, 72 samples and 72 controls were used. Analysis of *VEGF* polymorphism in Dengue patients was done using the rapid DNA extraction technique and Tetra-arms PCR. *Fig. 1* shows the *VEGF* gene polymorphism in Dengue patients. Tetra arms PCR utilized the annealing temperature of 67.3°C for amplification of *VEGF* gene (rs699947).

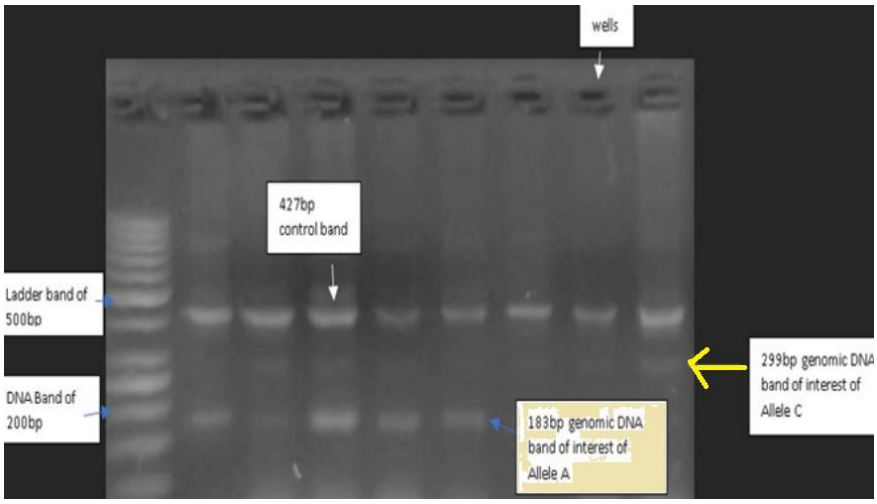

Figure 1. *Visualization of Genomic DNA bands from tetra-arm PCR of dengue samples*

Table 2 illustrates that the C allele was dominant among dengue cases and healthy controls. The *p-value* calculated depicts no genetically significant association between SNP genotype and phenotypic traits. Upon *in-silico* analysis, a variety of phytochemicals showed binding affinities with the target NS-1 protein (*Figure 2*). *Tables 3 and 4* enlist the top 5 phytochemicals showing antiviral properties out of 50, their ADME properties, and their binding affinities. Artemisinin showed the strongest binding affinity of -8.7 with target NS-1 protein while binding affinities of sanguinarine, chelidonine, glycyrrhisoflavone, and taspine were -8.5,-8.5,-8.1, and -7.8, respectively.

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Table 2. *SNP genotype and allele frequency in cases and controls for SNP rs699947*

Genotype	Proportion in cases (n=72)	Proportion in controls (n=72)	*P*-value
C/C	0.89	0.96	
A/A	0.06	0.01	
C/A	0.06	0.03	<0.001
C	0.92	0.97	
A	0.08	0.03	

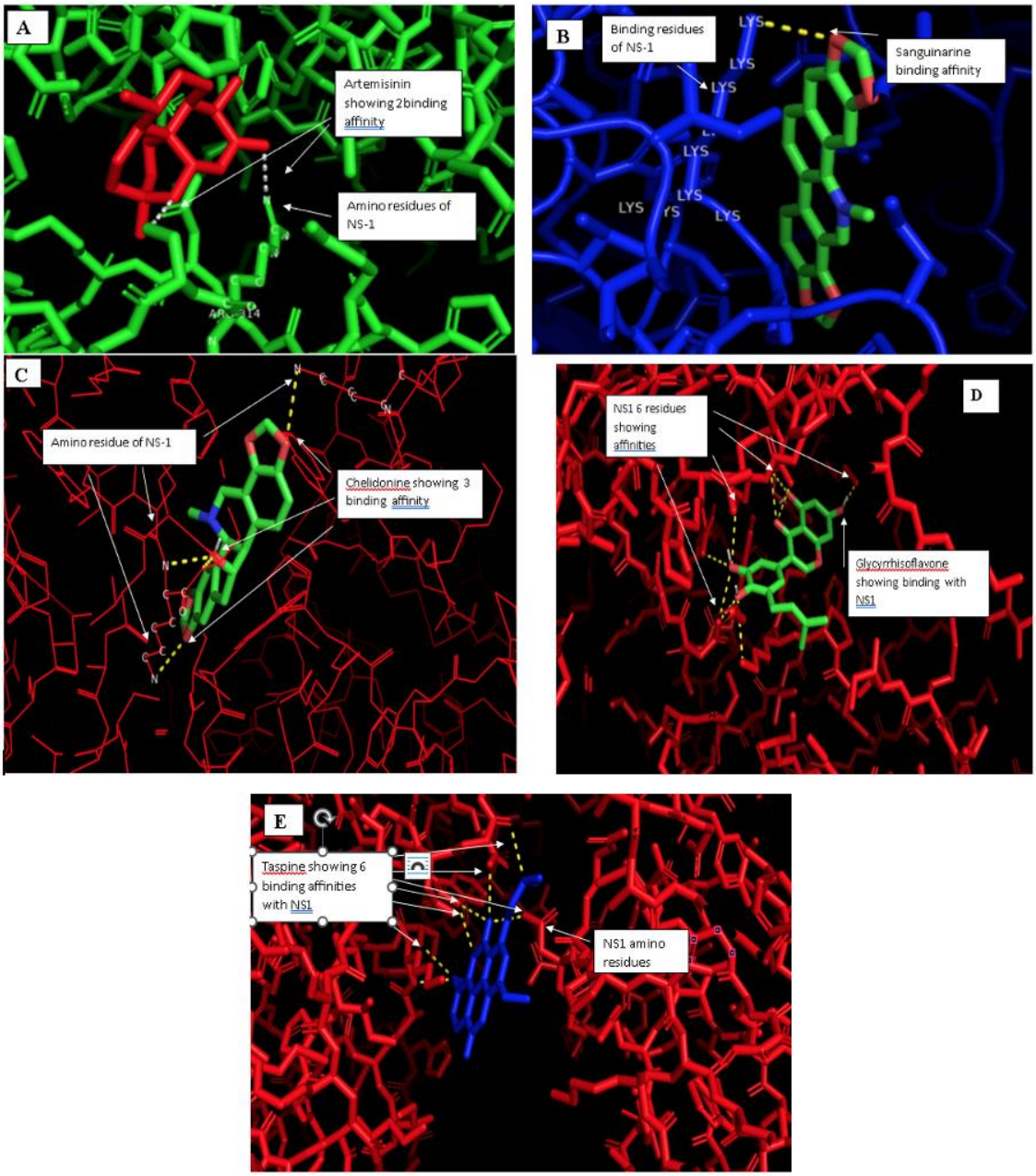

Figure 2. *Binding affinities of phytochemicals against DENV NS-1 protein: artemisinin (A), sanguinarine(B) chelidonine (C), glycyrrhisoflavone (D) and Taspine(E)*

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Table 3. *ADME properties of top 5 phytochemicals*

Compounds	Molecular weight (<500 Da)	H-bond acceptor (<10)	H-bond donor (<5)	Molar refractivity	Log P (<5)
Artemisinin	282.33 g/mol	5	0	70.38	2.75
Sanguinarine	332.33 g/mol	4	0	94.68	-0.04
Chelidonine	353.37 g/mol	6	1	96.12	3.2
Glycyrrhisoflavone	354.35 g/mol	6	4	99.73	2.56
Taspine	369.37 g/mol	7	0	102.49	3.10

Table 4. *NS-1 inhibitors and their binding affinities*

SR.no	Ligands	Configuration (PyMOL)	Binding Affinity
1.	Artemisinin	ARG 314 SER 315	-8.7
2.	SANGUINARINE	LYS 227	-8.5
3.	CHELIDONINE	LYS 206 LYS 227	-8.5
4.	GLYCYRRHISOFLAVONE	LYS 227 ASN 234 ARG 314 TRP 232 ASN 293 LYS 206	-8.1
5.	TASPINE	GLY 235 LYS 227 ILE 251 ASN 293 SER 228 TRP232	-7.8

Discussion

Female mosquitoes of the genus *Aedes aegypti* are the etiological agents for transmitting the Dengue virus to human hosts. Nearly 96 million people have this viral infection, widespread in over 100 countries, and asymptomatic (Ferrara, 2004; Naveed et al., 2024). When the dengue virus infects its host, it can induce undifferentiated nonfebrile or febrile infections, progressing to more serious cases of DHF or DSS. *VEGF* is essential in serious angiogenic and obsessive situations, such as malignancies, particularly infections involving plasma leakage (Hussain et al., 2023). The importance of *VEGF* in vascular angiogenesis has been highlighted in several studies, and defective angiogenesis can be caused by either losing the single *VEGF* allele or by abnormal cytokine release from damaged vascular endothelial cells, which could result in plasma leakage (Koukourakis et al., 2004). Finding the relationship between the *VEGF* rs699947 polymorphism and dengue patients was one of the objectives of this investigation. According to the findings, there was no appreciable change in allele frequency between patients and controls. Due to the small sample size, homozygous A alleles were only present in 3% of controls compared to 6% of samples, while the majority of samples

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(89%) and controls (96%) had homozygous C alleles. The prevalence of heterozygous alleles was only 6% in samples and 1% in controls. These results imply that samples and controls have equal chances of carrying the C allele.

The results of previous studies point to plasma leakage as the primary characteristic of DHF. Due to the limited sample size, lack of precision in data estimation, and absence of a meaningful connection between genotype and phenotypic features, our statistical analysis indicated a wide range of values of confidence intervals. Due to seasonal constraints, fewer samples were used in the study. Traditional plant-based therapies have been employed in Asia, Europe, and Africa in whole or in part, and this practice has persisted to this day. Because a dengue vaccine is antibody dependent, developing one is extremely challenging that would be effective against all four different serotypes responsible (Pintado Silva and Fernandez-Sesma, 2023).Further evidence that novel therapeutic alternatives and an effective next-generation vaccination, independent of earlier dengue exposure, should be developed to combat this 2,000-year-old disease was provided by the dengue vaccine's limited availability in some countries (Lim et al., 2021). As a result, plant based anti-DENV therapy may be a promising substitute for treating dengue. It was recognized that the antiviral characteristics prevented viral transcription, endocytosis, replication, and protein synthesis (Shu et al., 2023).

Finding antiviral phytochemicals that may specifically target the dengue virus protein NS1, an essential component of the viral replication complex, was the other goal of this research project. Dengue NS-1 performs a variety of tasks. It was chosen as the viral protein of interest. Among the phytochemicals artemisinin showed the highest affinity of -8.7 and exhibit strong antiviral properties. Intriguingly, sanguinarine, chelidonine, glycyrrhisoflavone, and taspine exhibit strong binding energies of -8.5, -8.5,-8.1 and -7.8 to the NS-1 viral protein, presenting exciting prospects for future drug development (Naveed et al., 2022a; Naveed et al., 2022b).

Conclusion

While significant strides have been made in understanding and combating dengue, its persistence as a global health threat necessitates sustained research endeavors. This study underscores the critical importance of expanding sample sizes and encompassing additional dengue serotypes, particularly DENV3 and DENV4, to comprehensively unravel this multifaceted disease's complex genetic and molecular landscape. Such insights hold the potential to catalyze the development of effective therapeutic strategies and preventive measures, ultimately mitigating the far-reaching impact of dengue on human populations.

Conflicts of Interest. No authors have disclosed any conflicts of interest.

Data Availability Statement. All the data produced or generated during the study has been given in the manuscript.

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