INTRODUCTION

Viruses are small infectious agents, which contain either RNA or DNA as genetic material, hence classified as RNA virus or DNA virus. The majority of the viruses have RNA genome, which is further classified into positive sense and negative sense RNA strands, and encodes limited number of proteins. The viral polypeptide is encoded by single open reading frame in all flaviviruses and cleaves into various proteins by cellular and viral proteases. A viral genome usually translates two kinds of proteins, i.e., structural proteins and nonstructural (NS) proteins. The structural proteins are involved in protection of the genome while the NS proteins are involved in the formation of viral replication complex.

The genus Flavivirus of the family Flaviviridae comprises large number of viruses. Most of these viruses are arthropod-borne human pathogens including Japanese encephalitis virus (JEV), West Nile virus (WNV), Yellow fever virus (YFV), Zika virus (ZIKV) and Dengue virus (DENV). Dengue is an arboviral infection and is transmitted primarily by Aedes aegypti mosquitoes which breeds in stagnant water. Dengue virus has five serotypes, namely DENV-1, DENV-2, DENV-3, DENV-4 and DENV-5. These are responsible for spectrum of diseases ranging from asymptomatic, mild febrile (dengue fever) to a life-threatening illness, dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS).

After the entry and attachment of virus with host cells, the progression of virus life cycle is carried out by the NS proteins of DENV. Nonstructural 4B is a trans-membrane protein and contributes to the inhibition of the interferon-α/β (IFN-α/β) response, therefore, plays a vital role in the virus replication and proliferation. It is the least targeted protein as compared to other proteins of dengue virus. NS3 dissociates NS4B from single stranded RNA by increasing the helicase activity. The structure of NS4B is 35% similar to other flavivirus, i.e., YFV and WNV, while the similarity of NS4B among dengue serotypes is 78–85%.

Nonstructural 4B protein from DENV-4 (DENV4-NS4B)
is highly hydrophobic and associates with the lumen side membrane of endoplasmic reticulum. The cytoplasmic loop and downstream C-terminal regions are specifically mediated for the dimerization of DENV4-NS4B.

Phytomedicines are naturally occurring compounds that have numerous medicinal properties. These are plant derived compounds, usually referred as phytochemicals. Several studies have reported the effectiveness of phytochemicals against various diseases. The phytochemicals are secondary metabolites produced in biosynthetic pathways of the plants, and there is a huge variety of these compounds which are known to have potential antiviral, antibacterial, antifungal, anticancer, and other properties.

Screening of drugs using in vitro and in vivo analysis is becoming increasingly difficult, time consuming and costly due to high number of compounds under investigation. The in silico approaches using computational techniques facilitate the drug discovery process by making the analysis cost-effective and resource efficient. More drugs can be discovered using the computational chemistry mechanism with minimal investment of money and time. Therefore, the main benefit of in silico drug design is its cost-effectiveness in research and development of drugs. Benefits of using in silico methods can be exploited in all the stages of drug development, i.e. from the preclinical discovery stage to late stage of clinical development. Computer aided drug design helps to screen the potent and most important medicinal compound with high efficiency. This in silico study targets the inhibition of DENV4-NS4B with phytochemicals derived from various medicinal plants, i.e. Silybum marianum, Tanacetum parthenium, Fumaria indica, Solanum nigrum, Andrographis paniculata and Melissa officinalis which are locally present in Pakistan and India. The phytochemicals of these plants are known to have inhibitory effect against many viral and bacterial diseases; however, this study analyses the inhibition potential of these phytochemicals against DENV4-NS4B.

MATERIAL & METHODS

Homology modelling

The study targeted the DENV4-NS4B protein for discovery of potential inhibitors against this protein; however, there is no crystal structure available for this protein. Due to this reason, homology modelling was performed to model the tertiary structure of the protein. NCBI-BLAST was used to find the homologous proteins of DENV4-NS4B and homology modelling was performed using Modeller 9.18.

Collection of phytochemicals

A total of 2750 phytochemicals including 1292 flavonoids, 488 sesquiterpene, 475 terpenoids and 495 alkaloids were selected through literature survey. It took 4–6 months for searching the plants and their phytochemicals. Search was made using different keywords such as plant names, their activities and country of origin (restricted to Pakistan and India only). For selected plants, their phytochemicals were searched and structures were retrieved from PubChem and DrugBank. These phytochemicals which have been reported for their antibacterial, antimi-crobial, antifungal, anti-tumor and antioxidative properties, were studied to identify their novel antiviral potential against dengue virus.

Screening of phytochemicals—ADMET and drug likeness prediction

The phytochemicals were filtered on the basis of ADMET properties and drug likeness prediction, using the PreADMET server. Pharmacological properties and pharmacokinetics of the phytochemicals, i.e. solubility (ESOL), gastro intestinal (GI) absorption, blood brain barrier (BBB) penetration and Lipinski’s rules violations were analyzed. The criteria set for screening compounds were: Lipinski’s violations = 0; Solubility = High; GI-absorption = High or Moderate; BBB-permeability = No; and Toxicity = Zero/Nil.

Molecular docking and binding energy estimation

Molecular docking of DENV4-NS4B with selected phytochemicals was performed using AutoDock Tools and Auto Dock Vina. Auto Dock Tools were used to prepare DENV4-NS4B model by the addition of polar hydrogen bonds which optimized the interactions between phytochemicals and DENV4-NS4B. A three-dimensional grid for DENV4-NS4B was designed with a size of $40 \times 56 \times 60$ Å³, to define the search space for phytochemicals to be docked against DENV4-NS4B. The phytochemicals (ligands) were also prepared for docking using same module with additional modification and torsion adjustment.

Density functional theory analysis

To study the reactivity and efficiency of the nine phytochemicals used against DENV4-NS4B, a density functional theory (DFT)-based analysis was carried out using highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) energy by applying the Becke, 3-parameter, Lee-Yang-Parr (B3LYP) correlation function of DFT. The band energy gap ($\Delta E$) was calculated using the expres-
RESULTS

Tertiary structure of DENV4-NS4B

DENV4-NS4B protein showed maximum similarity (74%) with DENV3-NS5 at the primary structure level, on performing BLAST analysis. The protein comprises of 172 residues arranged in 6 α-helices and 4 β-sheets. It is a highly hydrophobic transmembrane protein which is responsible for the membrane arrangements leading to the formation of viral replication complex, essential for the viral life cycle21.

ADMET results

All the phytochemicals were evaluated for ADMET properties and drug likeliness. Out of the 2750 phytochemicals, 1061 drugs were found to violate the Lipinski’s rule of five. Hence, the remaining 1689 phytochemicals were screened on the basis of BBB permeability, which showed that 756 were non-BBB permeable. Among the 756 phytochemicals, 650 showed high GI absorption, with optimum solubility. Toxicity and carcinogenic tests further screened the compounds and at last, out of total phytochemicals, 285 passed the criteria of being drug-like, having suitable ADMET profiles.

Docking of phytochemicals with DENV4-NS4B

All of the 285 phytochemicals were docked against DENV4-NS4B to calculate binding energy and inhibitory constant (K<sub>i</sub>) values. The docking results showed that all the phytochemicals expressed different behaviour while making interactions with the protein. For screening the best docked phytochemical, a threshold of –8 kcal/mol (binding affinity) was applied to the ligand-protein complexes. A total of nine phytochemicals showed binding affinity ≥ –8 kcal/mol, representing effective inhibition against DENV4-NS4B. These included Oxysanguinarine and Narlumicine from plant Fumaria indica; and Silymarin, Flavobion, Isosilybin, Mundulinol, Derrisin, Isopomiferin and Silydianin from Silybum marianum. All the seven phytochemicals from Silybum marianum were flavonoid in nature while the two from Fumaria indica were alkaloid (Tables 1–3).

Silymarin from Silybum marianum showed binding affinity of –9 kcal/mol (K<sub>i</sub> = 0.249 µM) and interacted with Ala<sub>4</sub>, Ile<sub>36</sub>, Ala<sub>128</sub>, Phe<sub>132</sub>, Leu<sub>134</sub> residues of DENV4-NS4B (Fig. 1a). Flavobion made interactions with Ala<sub>4</sub>, Ile<sub>36</sub>, Asn<sub>39</sub>, Ala<sub>128</sub>, Ala<sub>131</sub>, Phe<sub>132</sub>, and Asn<sub>137</sub> residues of DENV4-NS4B with binding affinity of –8.7 kcal/mol.

| Name of phytochemical | Molecular formula | Structural formula |
|-----------------------|------------------|-------------------|
| Silymarin             | C<sub>25</sub>H<sub>22</sub>O<sub>10</sub> |                     |
| Flavobion             | C<sub>25</sub>H<sub>22</sub>O<sub>10</sub> |                     |
| Isosilybin            | C<sub>25</sub>H<sub>22</sub>O<sub>10</sub> |                     |
| Mundulinol            | C<sub>25</sub>H<sub>26</sub>O<sub>5</sub> |                     |
| Derrisin              | C<sub>23</sub>H<sub>24</sub>O<sub>8</sub> |                     |
| Silydianin            | C<sub>23</sub>H<sub>24</sub>O<sub>8</sub> |                     |
| Isopomiferin          | C<sub>25</sub>H<sub>24</sub>O<sub>6</sub> |                     |
| Narlumicine           | C<sub>21</sub>H<sub>21</sub>No<sub>7</sub> |                     |
| Oxysanguinarine       | C<sub>20</sub>H<sub>13</sub>No<sub>5</sub> |                     |
Table 2. ADMET properties of the selected nine phytochemicals

| Molecule             | Estimated solubility log score | Estimated solubility class | Gastrointestinal absorption | Blood brain barrier penetration | Lipinski violations | Toxicity   | Carcinogenicity |
|----------------------|-------------------------------|---------------------------|-----------------------------|--------------------------------|---------------------|------------|----------------|
| Silymarin            | –3.44                         | Soluble                   | High                        | No                             | 0                   | Non-toxic  | Non-carcinogenic |
| Flavobion            | –4.14                         | Moderately soluble        | High                        | No                             | 0                   | Non-toxic  | Non-carcinogenic |
| Isosilybin           | –4.12                         | Moderately soluble        | High                        | No                             | 0                   | Non-toxic  | Non-carcinogenic |
| Mundulinol           | –3.85                         | Soluble                   | High                        | No                             | 0                   | Non-toxic  | Non-carcinogenic |
| Derrisin             | –2.85                         | Soluble                   | High                        | No                             | 0                   | Non-toxic  | Non-carcinogenic |
| Silydianin A         | –3.39                         | Soluble                   | High                        | No                             | 0                   | Non-toxic  | Non-carcinogenic |
| Isopomiferin         | –3.42                         | Soluble                   | High                        | No                             | 0                   | Non-toxic  | Non-carcinogenic |
| Fumaria indica       |                               |                           |                             |                                |                     |            |                |
| Narlumicine          | –3.65                         | Soluble                   | High                        | No                             | 0                   | Non-toxic  | Non-carcinogenic |
| Oxysanguinarine      | –3                            | Soluble                   | High                        | No                             | 0                   | Non-toxic  | Non-carcinogenic |

Table 3. Binding affinity, binding sites and Ki values of phytochemicals against DENV4-NS4B

| Phytochemical     | Binding affinity (kcal/mol) | Binding sites                                                                 | Ki (µM)  |
|-------------------|-----------------------------|------------------------------------------------------------------------------|----------|
| **Silybum marianum** |                             |                                                                              |          |
| Silymarin         | –9                          | Ala₄, Ile₃₅, Ala₁₂₈, Phe₁₃₂, Leu₁₃₄                                         | 0.249    |
| Flavobion         | –8.7                        | Ala₄, Ile₃₅, Asn₃₀, Ala₁₂₈, Ala₁₃₁, Phe₁₃₂, Asn₁₃₇                         | 0.413    |
| Isosilybin        | –8.7                        | Gly₃₅, Asn₃₀, Ala₁₂₈, Leu₁₃₀, Phe₁₃₂, Leu₁₃₄, Ile₁₃₅, Gln₁₃₉             | 0.413    |
| Mundulinol        | –8.8                        | Thr₉₃, Pro₉₅, Phe₁₃₂, Ile₁₃₅, Ile₁₃₆                                     | 0.349    |
| Derrisin          | –9.2                        | Thr₉₃, Leu₉₁, Asn₁₃₇, Gln₁₃₉, Thr₁₄₀                                     | 0.177    |
| Silydianin        | –9.4                        | Leu₉₀, Thr₁₀, Leu₁₃₁, Gly₁₀₅, Asn₁₃₇, Gln₁₃₉                             | 0.127    |
| Isopomiferin      | –9                          | Arg₃₁, Thr₁₃₂, Gly₃₅, Pro₁₀₅, Asn₁₃₇, Phe₁₃₂, Ile₁₃₅, Gln₁₃₉             | 0.249    |
| **Fumaria indica** |                             |                                                                              |          |
| Narlumicine       | –8.2                        | Leu₉₀, Leu₉₁, Pro₁₀₄, Gln₁₃₉, Thr₁₄₀                                     | 0.961    |
| Oxysanguinarine   | –8.2                        | Arg₃₁, Thr₁₃₂, Gly₃₅, Ala₁₂₈, Ala₁₃₁, Gln₁₃₉                              | 0.961    |

(Ki = 0.413 µM) (Fig. 1b). Isosilybin interacted with Gly₁₃₅, Asn₃₀, Ala₁₂₈, Leu₁₃₀, Phe₁₃₂, Leu₁₃₄, Ile₁₃₅, and Gln₁₃₉ residues at the binding cavity of DENV4-NS4B with binding energy –8.7 kcal/mol (Ki = 0.413 µM) (Fig. 1c). Mundulinol docked at Pro₉₅, Thr₉₃, Phe₁₃₂, Ile₁₃₅, and Ile₁₆ with binding energy –8.7 kcal/mol (Ki = 0.413 µM) (Fig. 1d). Derrisin made interactions with Thr₉₃, Leu₉₁, Asn₁₃₇, Gln₁₃₉ and Thr₁₄₀ with binding affinity –9.2 kcal/mol (Ki = 0.177 µM) (Fig. 1e). Silydianin made interactions at Leu₉₀, Thr₉₁, Gly₁₀₅, Asn₁₃₇, and Gln₁₃₉ with highest binding energy –9.4 kcal/mol (Ki = 0.127 µM) (Fig. 1f). Isopomiferin made interactions with Arg₃₁, Thr₁₃₂, Gly₃₅, Ile₁₃₅, Asn₁₃₇, Pro₁₀₅, Phe₁₃₂, Ile₁₃₅ and Gln₁₃₉ at the binding site of DENV4-NS4B with binding energy –9 kcal/mol (Ki = 0.249 µM) (Fig. 1g).

Narlumicine from *Fumaria*, with binding affinity of –8.2 kcal/mol (Ki = 0.961 µM), docked at Leu₉₀, Leu₉₁, Pro₁₀₄, Gln₁₃₉ and Thr₁₄₀ of DENV4-NS4B (Fig. 1h). Oxysanguinarine showed binding affinity of –8.2 kcal/mol (Ki = 0.961 µM) against DENV4-NS4B while making interactions with Arg₃₁, Thr₁₃₂, Gly₃₅, Ala₁₂₈, Ala₁₃₁, and Gln₁₃₉ residues (Fig. 1i).

**DFT and band energy gap results**

These results showed that the selected nine phytochemicals have effective reactivity, as they showed lower band gaps *i.e.* the difference of the E_LUMO and E_HOMO was low, ranging from 0.113 to 0.132 kcal/mol, implying the strong affinity of these inhibitors towards the target proteins. Among the nine phytochemicals, Silydianin from...
Fig. 1: Interactions of (a) Silymarin; (b) Flavobion; (c) Isosilybin; (d) Mundulinol; (e) Derrisin; (f) Silydianin; (g) Isopomiferin (from *Silybum marianum*); and (h) Narlumicine; (i) Oxysanguinarine (from *Furmia indica*) in the binding pocket of DENV4-NS4B.
The plants and their extracts have been used to cure diseases in humans since ancient times. Earlier studies have shown that phytochemicals act as good therapeutic agents to cure viral pathologies by targeting viral protein in host cells. These chemicals are found to be clinically safe for humans.

Lipinski’s rule of five is important rule for the evaluation of drug like properties of a compound which can be orally used in human for treatment against a disease. This rule deals with the appropriate number of hydrogen bonds of donor and acceptor, molecular weight and log P of the compound. In this study, the phytochemicals conforming to the Lipinski’s rule were further evaluated on the basis of BBB penetration behaviour. BBB is semipermeable membrane barrier which separates the circulating blood from the cerebrospinal fluid in the central nervous system (CNS) and the drug not reaching the CNS is considered to be more effective. Additionally, it was observed that the phytochemicals having non-penetrating behaviour also showed high GI absorption which is linked with epithelial cells and protects from drug absorption. Further, the phytochemicals showing optimum (high and moderate) solubility were only selected for analysis, which is considered an effective parameter in the drug discovery process.

The nine screened phytochemicals have been reported in various studies, for their inhibitory properties against different diseases. Out of these nine phytochemicals, five, i.e. Narlumicine and Oxysanguinarine from Fumaria Indica; Mundulinol, Derrisin and Isopomiferin from Silybum marianum are reported to be more effective against DEN4-NS4B, as the band energy gap was lowest among all the nine phytochemicals, i.e. 0.113 kcal/mol (Table 4).

| Phytochemicals | $E_{\text{LUMO}}$ (kcal/mol) | $E_{\text{HOMO}}$ (kcal/mol) | Band energy gap ($\Delta E$) (kcal/mol) |
|----------------|-------------------------------|-------------------------------|--------------------------------------|
| Silybum marianum |                               |                               |                                       |
| Silymarin      | $-0.121$                      | $-0.240$                      | 0.119                                |
| Flavobion      | $-0.127$                      | $-0.254$                      | 0.127                                |
| Isosilybin     | $-0.128$                      | $-0.255$                      | 0.127                                |
| Mundulinol     | $-0.124$                      | $-0.248$                      | 0.124                                |
| Derrisin       | $-0.117$                      | $-0.232$                      | 0.115                                |
| Silydianin     | $-0.110$                      | $-0.223$                      | 0.113                                |
| Isopomiferin   | $-0.120$                      | $-0.241$                      | 0.121                                |
| Fumaria indica |                               |                               |                                       |
| Narlumicine    | $-0.123$                      | $-0.254$                      | 0.131                                |
| Oxysanguinarine| $-0.125$                      | $-0.257$                      | 0.132                                |

**DISCUSSION**

Silybum marianum exhibited higher reactivity against DEN4-NS4B, as the band energy gap was lowest among all the nine phytochemicals, i.e. 0.113 kcal/mol (Table 4).

Silymarin has been studied for treatment of acetaminophen overdose injuries of the liver, kidney problems, improper working of cerebral cortex, histological changes and antioxidant activity. It has been also reported to reduce the effects of Aflatoxin B1 in bovine calves. Another study reported that extract of silymarin had inhibitory potential against hepatitis C virus in both, in vitro and in vivo. Antiviral efficacy of silymarin has also been reported against human papillomavirus 18, a highly carcinogenic virus. Silymarin has been used for the reduction glycemic level and progression of the complications related to diabetes which has been reported to enhances the rate of cardiovascular disease and kidney problems in Europe and United States. Kožurkova et al. have studied the effect of Flavobion in rat’s liver cells and reported that the change in concentration of Flavobion effects the regeneration of hepatocytes. Silydianin has been studied for the treatment of Aβ25-35-induced oxidative stress damage in HT-22 hippocampal cells which causes the Alzheimer’s disease. It was reported that Isosilybin reduces the production of reactive oxygen species (ROS), secretion of malondialdehyde and lactate dehydrogenase. In another study, Isosilybin has been identified as an effective drug candidate to prevent drug-drug interaction in cancer patients. Mundulinol has been reported to have cytotoxic properties against tumor cell line panel and is also reported to have antifungal potential as well.

Derrisin has been previously reported to have antibacterial activity against Helicobacter pylori. It has also been reported to reduce the oxidative metabolism of human polymorph nuclear neutrophils. Isopomiferin is known to have antioxidative properties. Narlumicine is an alkaloid in nature and has been reported as antifungal agent with potential results under field conditions. Oxysanguinarine has been studied against platelet aggregative constituents of Corydalis tashiroi.

In past, dengue virus has been targeted using phytochemicals from various plants. Various phytochemicals have been docked against NS4B, from each of the serotypes of DENV; wherein Catechin, Cianidanol, Epicat-echin, Eupatoretin, Glabranin, Laurifolin, DL-Catechin, are reported to have potential inhibitory property against dengue. It is reported that (–)-catechin, Epicat-echin and DL-Catechin are good antiviral agents against DENV-1, 2, and 4; however, none of these compound has been reported to be used against DENV3. Also, the binding affinities observed for the above compounds are very less,
These results are also in accordance with earlier reported studies\textsuperscript{45}—from –2.17 to –5.87 kcal/mol as compared to the present study which reflects the low efficacy and potential of those reported compounds\textsuperscript{43}.

In this study, band energy gaps ranged within 0.113 kcal/mol to 0.132 kcal/mol which is a narrow range and reflects high reactivity of compounds. It is well established in the literature that the lower band energy gap reflects higher reactivity of compounds since the $E_{\text{LUMO}}$ and $E_{\text{HOMO}}$ are responsible for charge transfer in a chemical reaction\textsuperscript{44}. These energies can also characterize the electrophilic or nucleophilic nature of a compound. These results are also in accordance with earlier reported studies\textsuperscript{45–47}.

**CONCLUSION**

This study was aimed at computer aided drug discovery against DENV-4 virus, targeting the NS4B protein. In the present study, out of 2750 phytochemicals from various medicinal plants, nine were screened for having strong binding affinity and effective drug likeness with suitable ADMET profiles. The DFT analysis also validated the reactivity of these compounds. It was found that Silydianin from *Silybum marianum* is the most reactive phytochemical among all the nine against DENV4-NS4B. Three phytochemicals from *Silybum marianum*, i.e. Derrisin, Mundaolin and Isopomiferin, and two phytochemicals, i.e. Narlumicine and Oxysanguinarine from *Fumaria indica* were reported for first time as novel anti-dengue leads/agents. These phytochemicals can be further used for *in vitro* and *in vivo* analyses for determining their efficacy and safety as drugs against DENV in humans. The development of phytochemicals as potential drugs for DENV would be therapeutically and economically feasible.

**Conflict of interest**

The authors declare no conflict of interest.

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